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Suppression of High-Pressure-Induced Hemolysis of Human Erythrocytes by Preincubation at 49°C[1]

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When human erythrocytes were preincubated at 37–52°C under atmospheric pressure before exposure to a pressure of 200 MPa at 37°C, the value of hemolysis was constant (about 43%) up to 45°C but became minimal at 49°C. The results from anti-spectrin antibody–entrapped red ghosts, spectrin-free vesicles, and *N*-(1-pyrenyl)iodoacetamide-labeled ghosts suggest that the denaturation of spectrin is associated with such behavior of hemolysis at 49°C. The vesicles released at 200 MPa by 49°C-preincubated erythrocytes were smaller than those released by the treatment at 49°C or 200 MPa alone. The size of vesicles released at 200 MPa was independent of preincubation temperature up to 45°C, and the vesicles released from 49°C-preincubated erythrocytes became smaller with increasing pressure up to 200 MPa. Thus, hemolysis and vesiculation under high pressure are greatly affected by the conformation of spectrin before compression. Since spectrin remains intact up to 45°C, the compression of erythrocytes at 200 MPa induces structural changes of spectrin followed by the release of large vesicles and hemolysis. On the other hand, in erythrocytes that are undergoing vesiculation due to spectrin denaturation at 49°C, compression produces smaller vesicles, so that the hemolysis is suppressed.

Key words: hemolysis, high pressure, human erythrocyte, spectrin, vesicle.

The membrane structure of human erythrocytes has been extensively studied as a prototype of the biological membrane. The shape, stability, and deformability of the erythrocytes are controlled by membrane–cytoskeleton interactions (*1*). Human erythroid spectrin is a major component of cytoskeletal proteins, which associates with such transmembrane proteins as band 3 and glycophorin C *via* ankyrin (*2–4*) and protein 4.1 (*2–5*), respectively Spectrin is a heterodimer of α and β subunits with calculated molecular masses of 280 (*6*) and 246 kDa (*7*), respectively. The spectrin subunits are arranged side-to-side in antiparallel fashion into a rod-like dimer. Analysis of the primary sequence of spectrin showed that the α and β subunits contain about 20 and 17 segments of a 106 amino acid repeating motif, respectively (*8*). Spectrin heterodimers self-associate head-to-head to form tetramers and oligomers (*9, 10*). Mutations affecting the equilibrium between spectrin dimers and tetramers result in hereditary elliptocytosis (*11*). On the other hand, a deficiency of α- and β-spectrin results in hereditary spherocytosis (*11*).

We have hitherto examined the membrane structure of human erythrocytes exposed to high pressure. High-pressure-induced hemolysis starts to occur at about 130 MPa, and the value of hemolysis at 200 MPa is about 43% (*12*). In such hemolyzed cells, spectrin is partially detached from the membrane (*13*) Upon chemically cross-linking spectrin and actin with transmembrane proteins such as band 3 and glycophorins, high-pressure-induced hemolysis is almost completely suppressed (*12*). In this case, no detachment of spectrin is observed. Hemolytic properties at 200 MPa of *N*-ethylmaleimide–treated erythrocytes are also associated with the reorganization of cytoskeleton brought about by the conformational changes of spectrin (*14*). Thus, it seems likely that spectrin plays an important role in high-pressure-induced hemolysis. To analyze in detail the role of spectrin, the effect of pressure on the membrane structure of heated erythrocytes has been examined. It is well known that spectrin is denatured above 49°C (*15*). In the present work, we demonstrate that high-pressure-induced hemolysis and vesiculation of erythrocytes are affected by the thermal denaturation of spectrin at 49°C.

MATERIALS AND METHODS

Chemicals—Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma. *N*-(1-pyrenyl)iodoacetamide (NPIA) was purchased from Molecular Probes. All other chemicals were of reagent grade.

High-Pressure-Induced Hemolysis of Heated Erythrocytes, Red Ghosts, and Vesicles—Human blood samples were obtained from the Fukuoka Red Cross Blood Center. Blood samples were centrifuged at 750 ×*g* for 10 min at 4°C. The plasma and buffy coat were carefully removed. The erythrocytes were washed three times in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), suspended at 10% hematocrit in PBS, then incubated for 15 min at 37–52°C under atmospheric pressure. All treatments except for

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Abbreviations DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; NPIA, *N*-(1-pyrenyl)iodoacetamide, PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4

high pressure were performed under atmospheric pressure unless otherwise noted. After incubation, the erythrocytes were washed twice with PBS. To examine whether the formation of disulfide bonds is associated with the hemolytic properties under high pressure of heated erythrocytes, 49°C-treated erythrocytes (10% hematocrit) in PBS were treated with 10 mM dithiothreitol (DTT) for 30 min at 37°C, then washed twice in PBS.

Anti-spectrin antibody-entrapped red ghosts or anti-spectrin antibody-free ones were prepared as follows. Anti-spectrin antibody was isolated from rabbit antiserum using a spectrin-sepharose 4B column and its identity was confirmed by Western blot analysis. Intact erythrocytes were hemolyzed in 4 volumes of 5 mM sodium phosphate buffer (pH 8.0) with or without anti-spectrin antibody (2.0 mg/ml) at 0°C for 10 min. The hemolyzates were incubated for 10 min at 37°C, made isotonic (final concentration: 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$), and incubated to reseal the membranes for 1 h at 37°C. These red ghosts were washed several times in PBS, suspended in 100 volumes of PBS, and exposed to 49°C for 15 min.

To prepare spectrin-free vesicles, the erythrocytes (10% hematocrit) were incubated with DMPC liposomes (0.74 mM) in PBS for 10 h at 37°C (*16*). After incubation, the suspensions were centrifuged at 1,800 ×*g* for 10 min at room temperature to remove the erythrocytes The supernatants were filtered using a white plane Millipore filter (SS type for pore size 3 μm) to avoid contamination by ghosts The same type of filter was used for all filtrations unless stated otherwise. The filtrates containing vesicles were centrifuged at 18,000 ×*g* for 30 min at 4°C. The pellets were washed in PBS. The vesicles were suspended in 100 volumes of PBS and exposed to 37 or 49°C for 15 min

For high-pressure-induced hemolysis, the erythrocytes, red ghosts, or vesicles were suspended in PBS and exposed to a pressure of 200 MPa for 30 min at 37°C, as previously described (*13*). Unless otherwise noted, all high-pressure treatments were performed at 37°C for 30 min. After decompression, the suspensions were centrifuged at 35°C for 10 min at 750, 1,800, or 18,000 ×*g* for erythrocytes, red ghosts, or vesicles, respectively. The absorbance of the supernatant was measured at 542 nm. One hundred percent hemolysis was achieved by adding 0.3% volume of 5% Triton X-100 into the suspension For hypotonic hemolysis, the erythrocytes (10 μl) were added to 3 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 54 mM NaCl, incubated for 10 min at 37°C, and then centrifuged for 5 min at 750 ×*g*. The degree of hemolysis was similarly determined.

Excimer Fluorescence in Pyrene-Labeled Ghosts—White ghosts were prepared by hemolyzing intact erythrocytes in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0), then washing the cells repeatedly in the same buffer, according to the method of Dodge *et al.* (*17*). These ghosts were incubated in PBS for 1 h at 37°C to reseal their membranes. The resealed ghosts in 10 volumes of PBS were incubated for 15 min at 37, 45, 49, and 52°C, or exposed to a pressure of 200 MPa. The ghosts were also preincubated for 15 min at 49°C before exposure to 10 mM DTT for 30 min at 37°C to reduce disulfide bonds in membrane proteins or to a pressure of 200 MPa. These ghosts were washed twice in PBS. Such ghosts were suspended in 10 volumes of PBS, incubated with NPIA (10 μg/ml) at 0°C for 17 to 23 h, then centrifuged at 18,000 ×*g* for 20 min at 4°C. The pellets were washed several times with PBS. One volume of 20% Triton X-100 was added to 19 volumes of NPIA-labeled ghost suspension The suspensions were incubated for 1 h at 0°C and centrifuged at 18,000 ×*g* for 20 min at 4°C. The supernatants and Triton shells suspended in PBS were used for the fluorescence measurement. For the measurement of fluorescence, the protein concentrations of ghosts, Triton supernatants, and Triton shells were about 150, 100, and 30 μg/ml, respectively. Fluorescence spectra of NPIA were measured using a model FP-750 spectrometer (JASCO, Japan) with excitation at 345 nm (slit width, 5 nm).

Extraction of Cytoskeletal Proteins from 49°C-Treated Ghosts and SDS-PAGE of the Ghosts—The resealed white ghosts in 10 volumes of PBS were incubated for 15 min at 37 or 49°C. Portions of 49°C-preincubated ghosts were treated with 10 mM DTT for 30 min at 37°C and washed twice in PBS To extract cytoskeleton, these ghosts were washed once in chilled 0.1 mM EDTA buffer (pH 8.0) and suspended in 10 volumes of same buffer. The ghost suspensions were incubated for 1 h at 37°C and centrifuged at 18,000 ×*g* for 30 min at 4°C The protein concentrations of the supernatants were determined by the method of Lowry *et al.* (*18*). Ghosts were also prepared from 49°C (15 min)- and/or 200 MPa–treated erythrocytes, as described above (*17*). SDS-PAGE of these ghosts was performed on 5% gel in the presence or absence of 2-mercaptoethanol, according to the method of Laemmli (*19*). Gels were stained with Coomassie Blue.

Size of Vesicles—The membrane surface of erythrocytes exposed to 49°C, 200 MPa, and 49°C/200 MPa was examined under a scanning electron microscope. Erythrocyte suspensions (10% hematocrit) in PBS were exposed to 49°C for 15 min, 200 MPa, or 49°C for 15 min and then 200 MPa, then centrifuged at 750 ×*g* for 10 min. The pellets were suspended in PBS containing 0.5% bovine serum albumin and fixed with 1% glutaraldehyde at 20°C for 2 h, as described previously (*20*). The samples were dried with a Hitachi critical point dryer (model HCP-2), then coated with gold using a Bio-Rad sputtering outfit (model E5100). A Hitachi S-450LB scanning electron microscope was used. The supernatants of the treated erythrocyte suspensions were filtered. The sizes of vesicles in filtrates were measured at room temperature (about 21°C) by using a submicron particle sizer (model 370, NICOMP, Calif., USA) with a laser wavelength of 488 nm.

To examine the effect of preincubation temperature on size of vesicles released at 200 MPa, the erythrocytes in PBS at 10% hematocrit were incubated at 37, 40, 45, and 49°C for 15 min, then washed twice in PBS. These heat-treated cells were exposed to a pressure of 200 MPa. After decompression, the suspensions were centrifuged at 750 ×*g* for 10 min at 35°C. The supernatants were filtered, and the size of vesicles in filtrates was determined by the light-scattering measurement, as mentioned above.

To examine the effect of high pressure on release and size of vesicles, the erythrocytes were preincubated at 49°C for 15 min, suspended in PBS at 10% hematocrit, then exposed to various pressures (0.1, 40, 80, 100, 130, 160, and 200 MPa). After decompression, the suspensions were centrifuged at 750 ×*g* for 10 min at 35°C and the supernatants were filtered. The turbidity and the size of vesicle in fil-

trates were determined by measuring absorbance at 650 nm and light scattered at 488 nm, respectively.

Spectrin Content in Vesicles—Erythrocytes suspended at 10% hematocrit in PBS were preincubated for 15 min at 37, 40, 45, or 49°C, then exposed to a pressure of 200 MPa After decompression, the suspensions were centrifuged at 1,800 ×*g* for 10 min at room temperature. The supernatants were filtered, and the filtrates were centrifuged at 18,000 ×*g* for 20 min at 4°C. To analyze the membrane protein composition in vesicles, SDS-PAGE of membrane proteins was performed on 8% gel, as mentioned above. The stained bands were analyzed using an Advantec DM-303 scanning densitometer. The spectrin content in the vesicles was evaluated from the ratio of band intensity of spectrin to band 3.

RESULTS

High-Pressure-Induced Hemolysis Is Most Suppressed by Preincubation of Erythrocytes at 49°C—Human erythrocytes were preincubated for 15 min at various temperatures (37–52°C) under atmospheric pressure, then exposed to a pressure of 200 MPa or a hypotonic buffer. The value of hemolysis at 200 MPa remained almost constant (about 43%) up to 45°C, began to decrease at 47°C, and was minimal at 49°C (Fig. 1). When the erythrocytes preincubated at 49°C were further treated with DTT, the value of hemolysis at 200 MPa remained unchanged (15.7%). The value of hemolysis at 200 MPa increased upon preincubation at 52°C as compared with 49°C In 52°C-preincubated erythrocytes, a small degree of hemolysis was observed. However, when the erythrocytes were exposed to 52°C for 5 min, no hemolysis due to preincubation was observed, and the value of hemolysis at 200 MPa of such cells was about 15.9%. This value is close to that of the erythrocytes preincubated for 15 min at 49°C. Therefore, these results suggest that the value of hemolysis at 200 MPa in heated erythrocytes is dependent on the degree of membrane damage induced by heating. Furthermore, when erythrocytes preincubated at various temperatures were exposed to hypotonic buffer, the value of hypotonic hemolysis changed in a fashion similar to that of high-pressure-induced hemolysis (Fig. 1). However, the degree of suppression at 49°C was smaller than in the case of high pressure

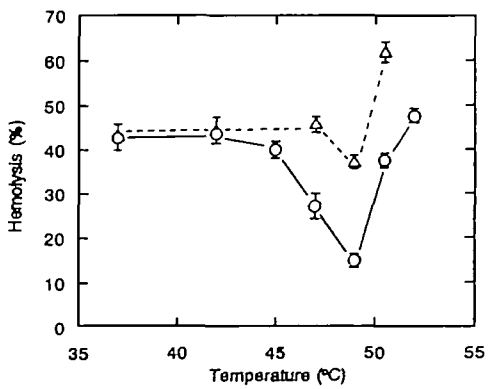

Fig. 1 **Effects of preincubation temperature on hemolysis under high pressure or hypotonic buffer.** The erythrocytes in PBS were preincubated at various temperatures (37, 42, 45, 47, 49, 50.5, and 52°C) for 15 min under atmospheric pressure and washed twice in PBS These erythrocytes were exposed to a pressure of 200 MPa (○) for 30 min at 37°C or hypotonic buffer (△) for 10 min at 37°C Values are means ± SD for three experiments.

Hemolytic Properties at 200 MPa of 49°C-Preincubated Erythrocytes Are Associated with Spectrin—We previously demonstrated that spectrin is partially detached from the membrane in 200 MPa–treated erythrocytes (*13*). The cross-linking of spectrin by anti-spectrin antibody may stabilize the spectrin network against high pressure at 200 MPa or heating at 49°C. So, anti-spectrin antibody–entrapped red ghosts and anti-spectrin antibody–free ones were preincubated at 37°C and subjected to a pressure of 200 MPa. In this case, high-pressure-induced hemolysis was greatly suppressed by anti-spectrin antibody (Table I). As with erythrocytes, hemolysis at 200 MPa of anti-spectrin antibody–free red ghosts was also suppressed upon preincubation at 49°C. On the other hand, the hemolysis at 200 MPa of anti-spectrin antibody–entrapped red ghosts was not suppressed by the preincubation at 49°C.

To characterize the hemolytic behavior at 200 MPa of 49°C-preincubated erythrocytes, we attempted to use spectrin-free vesicles Spectrin-free vesicles contain transmembrane proteins such as band 3 and glycophorins, but not cytoskeletal proteins such as spectrin and actin (*21, 22*). Such vesicles are released into the buffer from the erythrocyte membrane surface when the erythrocytes are incubated with DMPC liposomes. Spectrin-free vesicles thus prepared were preincubated at 37 or 49°C, then exposed to a pressure of 200 MPa. However, the hemolysis at 200 MPa of these vesicles was not suppressed by the preincubation at 49°C (Table I).

Structural Changes in Cytoskeleton of Erythrocytes Are Induced by Heating at 49°C and/or High Pressure of 200 MPa—To analyze hemolytic properties of 49°C-preincubated erythrocytes at 200 MPa, resealed white ghosts were exposed to various temperatures (37–52°C) and/or 200 MPa, and then labeled with NPIA, a SH group-reactive agent. Excimer signals of pyrene were observed at around 474 nm in ghosts treated above 49°C (Fig. 2A), or at 49°C and/or 200 MPa (Fig. 2B). This excimer fluorescence was not affected by DTT treatment of 49°C-preincubated ghosts (data not shown). Further, NPIA-labeled ghosts in Fig. 2B were treated with 1% Triton X-100 and centrifuged. The excimer signals were observed not in the supernatant (Fig. 2C) but in the Triton-shell (Fig. 2D). To characterize the membrane structure of 49°C-treated erythrocytes, resealed white ghosts were preincubated at 49°C, then exposed to a low ionic strength buffer (0.1 mM EDTA, pH 8.0). The amounts of cytoskeletal proteins extracted from 37- and

TABLE I. **Hemolytic properties at 200 MPa of 49°C-preincubated anti-spectrin antibody–entrapped red ghosts and spectrin-free vesicles.** The samples were prepared as described in "MATERIALS AND METHODS" and preincubated for 15 min at 37 or 49°C before exposure to a pressure of 200 MPa for 30 min at 37°C Values are means ± SD for at least two experiments.

	% Hemolysis at 200 MPa	
	Preincubation	
	37°C	49°C
Intact erythrocytes	42 7 ± 3.1	14.8 ± 0.5
Anti-spectrin antibody–free red ghosts	50 5 ± 1 5	22 8 ± 4 1
Anti-spectrin antibody–entrapped red ghosts	12 1 ± 2 3	20 6 ± 3 2
Spectrin-free vesicles	31 8 ± 3.9	40 4 ± 3 9

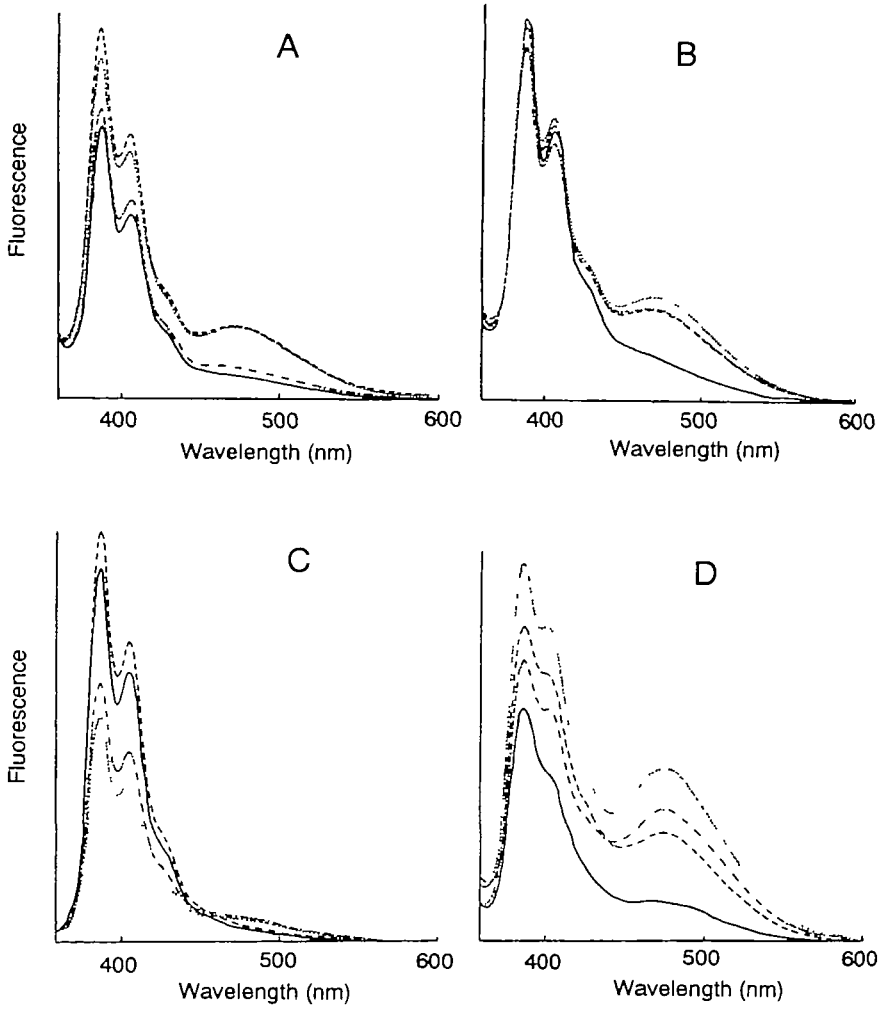

Fig 2 **Fluorescence spectra of NPIA-labeled ghosts.** In panel A, resealed white ghosts in PBS were incubated at 37 (——),45 (– –), 49 (– – –), or 52°C (·) for 15 min at atmospheric pressure In panel B, resealed white ghosts were treated as follows ——, treatment at 37°C for 15 min at atmospheric pressure; – – –, treatment at 49°C for 15 min at atmospheric pressure, —–, exposure to 200 MPa for 30 min at 37°C, ·····, treatment at 49°C for 15 min at atmospheric pressure and then 200 MPa for 30 min at 37°C These ghosts (A and B) were labeled with NPIA for 17–23 h at 0°C NPIA-labeled ghosts in panel B were treated with 1% Triton X-100 and centrifuged. The fluorescence spectra of the supernatants (C) and pellets suspended in PBS (D) were observed.

49°C-treated ghosts were 33.6 and 19.7%, respectively. When the 49°C-incubated ghosts were treated with 10 mM DTT, the amounts of cytoskeletal proteins extracted by 0.1 mM EDTA (pH 8.0) remained unchanged (21.1%). Furthermore, SDS-PAGE under unreduced conditions of ghosts prepared from 49°C- and/or 200 MPa-treated erythrocytes showed high-molecular-weight bands on the top of the gel in all samples (data not shown). These bands disappeared under reduced conditions.

Small Vesicles Are Released upon Exposure of 49°C-Preincubated Erythrocytes to a Pressure of 200 MPa—The membrane surface of heated and/or compressed erythrocytes was examined by scanning electron microscopy (Fig. 3). Upon exposure to a pressure of 200 MPa, vesicles of various sizes were formed on the membrane surface of erythrocytes (Fig. 3, A and B). Similar vesicles were observed on the membrane surface of 49°C-treated erythrocytes (Fig. 3C). Interestingly, when 49°C-pretreated erythrocytes were exposed to a pressure of 200 MPa, the vesicles formed appeared to be smaller than in the case of treatment at 49°C or 200 MPa alone (Fig. 3D).

Parts of vesicles seen in Fig. 3 are detached from the membrane surface of the erythrocyte (*20*). The sizes of released vesicles can be determined from light-scattering measurements. When erythrocytes preincubated at 37, 40, or 45°C, at which temperatures no vesicles were released by the incubation, were subjected to a pressure of 200 MPa, the diameter of released vesicles was about 460 nm in all cases (Fig. 4). On the other hand, vesicles of about 390 nm in diameter were released from 49°C-incubated erythrocytes. Upon exposure of 49°C-preincubated erythrocytes to a pressure of 200 MPa, vesicles of about 260 nm in diameter were released (Fig. 4).

Small Vesicles Contain Less Spectrin—The membrane protein compositions of the vesicles released by high pressure were analyzed by SDS-PAGE. The vesicles released from 37°C-preincubated erythrocytes by a pressure of 200 MPa show a band pattern similar to that of the erythrocyte membrane, although band intensities in a few bands are different (*20*). In vesicles released by 200 MPa from 37, 40, or 45°C-preincubated erythrocytes, the contents of spectrin relative to band 3 were almost the same (Fig. 4). On the other hand, the high-pressure-induced vesicles from erythrocytes preincubated at 49°C showed decreased spectrin contents (Fig. 4).

Effects of High Pressure on Vesiculation of 49°C-Treated Erythrocytes—To examine the effect of high pressure on vesiculation, the 49°C-preincubated erythrocytes were washed and exposed to various pressures (0.1–200 MPa) (Fig. 5). The vesicle release was accelerated under high pressure. The diameter of released vesicles decreased from 490 to 260 nm with increasing pressure from 0.1 to 200

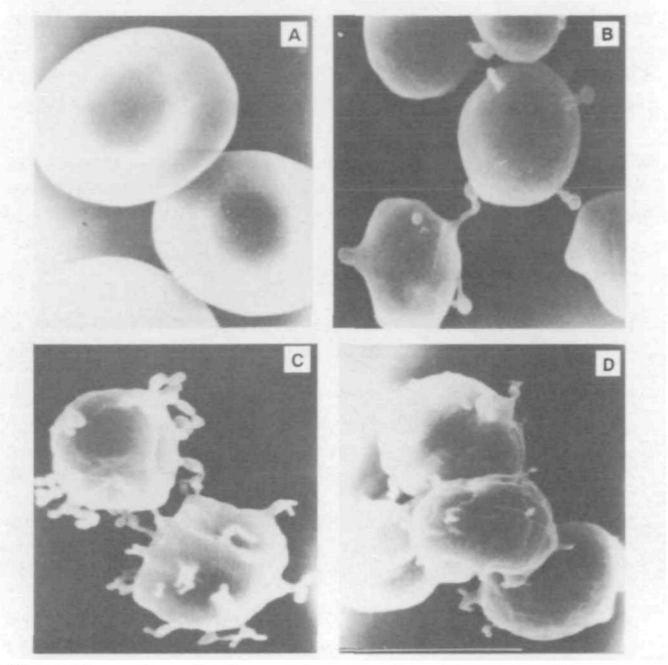

Fig 3 **Scanning electron micrographs of heat- and/or pressure-treated erythrocytes.** (A) intact cells, (B) cells exposed to 200 MPa for 30 min at 37°C, (C) cells incubated at 49°C for 15 min at atmospheric pressure, (D) cells exposed to 49°C for 15 min at atmospheric pressure and then 200 MPa for 30 min at 37°C Bar, 5 μm.

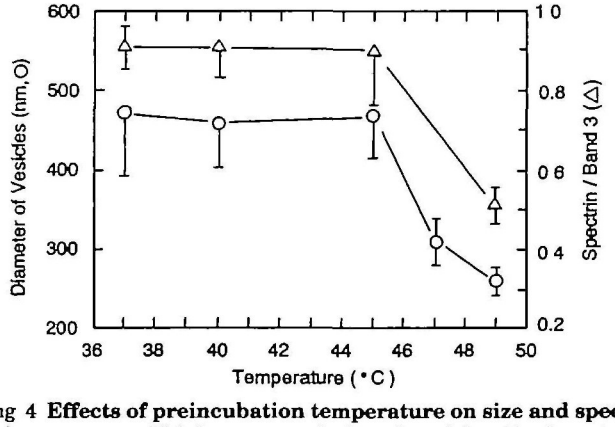

Fig 4 **Effects of preincubation temperature on size and spectrin contents of high-pressure-induced vesicles.** Erythrocytes were preincubated at various temperatures for 15 min under atmospheric pressure and washed in PBS These cells were exposed to 200 MPa for 30 min at 37°C. After decompression, membrane vesicles were prepared by centrifugation and filtration The size of vesicles was determined by light scattering. Membrane proteins of vesicles were separated by SDS-PAGE and quantitated using a densitometer The ratio of band intensities of spectrin and band 3 is given Values are means ± SD for at least three experiments.

MPa. In this case, the vesicles released under atmospheric pressure were larger than those (390 nm) released by incubation of erythrocytes at 49°C. This difference may be ascribed to the different temperatures (37 and 49°C) during vesiculation and to washing of heated cells.

DISCUSSION

In the present work, we have demonstrated that high-pressure-induced hemolysis is largely suppressed by the preincubation of the erythrocytes at 49°C. Several lines of evidence show that spectrin undergoes structural changes above 49°C. For example, a shift from the tetramer to the dimer, reduced binding of heated spectrin to normal inside-out vesicles, and changes in the spectrin tryptophan fluorescence have been reported (*23*). Upon NPIA labeling of ghosts heated above 49°C, excimer fluorescence of pyrene is observed. This excimer fluorescence is observed in the Triton shell composed primarily of spectrin and actin (*24*). Judging from the concentration ratio of these proteins in the Triton shell and the number of SH-groups in each protein (*6, 7*), it seems likely that the excimer fluorescence in NPIA-labeled ghosts is mostly due to spectrin (*25*). It is known that the excimer fluorescence of pyrene appears when the molecules are located within 2.4 Å each other. Thus, in ghosts heated above 49°C, the structural changes induced in spectrin bring the SH groups in spectrin closer together. Such an excimer fluorescence appeared in 200 MPa–treated ghosts, suggesting that exposure to this pressure can also induce the structural changes of spectrin. Interestingly, the suppression of hemolysis at 200 MPa seen in 49°C-preincubated erythrocytes and anti-spectrin antibody–free red ghosts is not seen upon heating spectrin-free vesicles and anti-spectrin antibody–entrapped red ghosts at 49°C. Taken together, these results suggest that the suppression of high-pressure-induced hemolysis in 49°C-preincubated erythrocytes is associated with the conformational changes, *i.e.*, denaturation, of spectrin. SDS-PAGE of ghosts prepared from 49°C-treated erythrocytes shows the high-molecular-weight band on the top of the gel (*23*). This band disappears under reduced conditions. On the other hand, the suppression of hemolysis at 200 MPa, the low extractability of cytoskeleton by a low ionic strength buffer, and the appearance of excimer fluorescence in 49°C-treated erythrocytes or ghosts are unaffected under reduced conditions. Thus, the suppression of high-pressure-induced

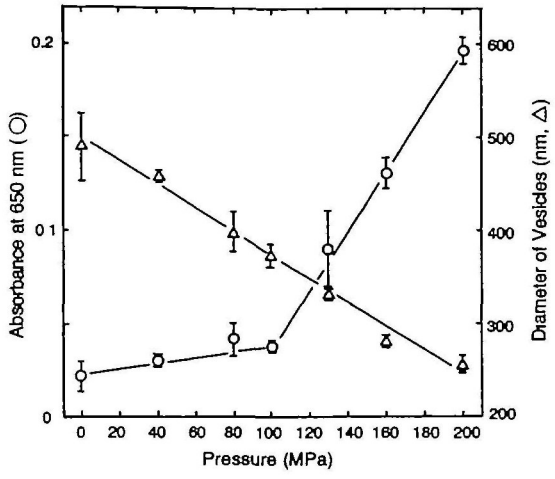

Fig 5 **Effects of high pressure on vesicle release from 49°C-preincubated erythrocytes.** The erythrocytes preincubated for 15 min at 49°C were washed twice, suspended in PBS, and exposed to pressures from 0 1 to 200 MPa for 30 min at 37°C After decompression, the suspensions were centrifuged at 750 ×*g* for 10 min at 35°C. The supernatants were filtered through a filter (3 μm). The turbidity at 650 nm and the size of vesicles in filtrates were measured. Values are means ± SD for at least two experiments

hemolysis in 49°C-preincubated erythrocytes is independent of the formation of such a high-molecular-weight polymer

There are several reports concerning vesicles released from erythrocytes. For instance, all vesicles released from erythrocytes by ATP depletion (*26*), DMPC liposome (*16, 21 22*), and Ca^{2+}-loading (*27*) are about 180 to 220 nm in diameter and spectrin-free, although integral proteins such as band 3 and glycophorins are present. These vesicles are released without hemolysis. Upon heating of erythrocytes at 49°C, the denaturation of spectrin occurs so that membrane vesicles are released without hemolysis (*28*). Released vesicles are about 390 nm in diameter and spectrin-poor in the protein composition (*29*). On the other hand, when intact erythrocytes are exposed to a pressure of 200 MPa, both hemolysis and vesiculation take place (*20*). The size of released vesicles is about 460 nm. High-pressure-induced vesicles contain considerable amounts of spectrin as well as other membrane proteins (*20*). These data suggest that the size of vesicles released from the erythrocytes is dependent on the content of spectrin within vesicles It is of interest to consider the effect of high pressure and heating on vesicle size. The size of vesicles released from 49°C-treated erythrocytes decreases with increasing pressure. In general, the volume of substances decreases with increasing pressure, although isothermal compressibilities vary between the liquid and solid states. Therefore, it seems likely that the cell volume decreases under pressure. The present results demonstrate that the size of vesicles released under high pressure from heated erythrocytes is explicable in terms of this pressure–volume relationship On the other hand, the vesiculation of erythrocytes at 200 MPa is also affected by preheating the cells. The size of membrane vesicles released by a pressure of 200 MPa remains constant (about 460 nm) irrespective of preincubation temperature up to 45°C, but decreases to about 260 nm at 49°C. This suggests that the size of high-pressure-induced vesicles is also decreased by the thermal denaturation of spectrin. In 49°C-treated ghosts, the extraction of cytoskeleton from the membrane by a low ionic strength buffer is suppressed by reorganization of cytoskeleton due to spectrin denaturation (*23*). The decrease of spectrin content in vesicles released from 49°C-preincubated erythrocytes at 200 MPa may also be ascribed to such reorganization of the cytoskeleton.

The present data demonstrate that the erythrocyte membrane behaves differently under high pressure depending on whether spectrin is denatured before compression. In the case of native spectrin, the destruction of cytoskeleton by high pressure induces the release of large vesicles and hemolysis. Such large vesicles contain large amounts of spectrin (*20*). Therefore, it seems likely that the release of large vesicles results in severe damage to the cytoskeletal structure of the parent cell. When these pressure-treated erythrocytes are incubated at 0°C under atmospheric pressure, hemoglobin is released from the membrane (*13*). This suggests that the pressure-induced membrane holes also exist after decompression. On the other hand, the thermal denaturation of spectrin induces the vesicles on the membrane surface. Upon exposure of such cells to a pressure of 200 MPa, smaller vesicles with lower contents of spectrin are released and the hemolysis is suppressed In this case, the damage to the cytoskeleton induced by high pressure may be less than in the case of intact erythrocytes. Thus, the formation of smaller membrane holes is expected. These results suggest that the sizes of released vesicles play an important role in high-pressure-induced hemolysis.

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REFERENCES

1. Branton, D, Cohen, C M, and Tyler, J (1981) Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell* **24**, 24–32
2 Tyler, J.M, Hargreaves, W R, and Branton, D (1979) Purification of two spectrin-binding proteins: Biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4 1 *Proc. Natl Acad Sci USA* **76**, 5192–5196
3. Low, P S, Willardson, B M, Mohandas, N., Rossi, M., and Shohet, S (1991) Contribution of the band 3-ankyrin interaction to erythrocyte membrane mechanical stability *Blood* **77**, 1581–1586
4 Michaely, P and Bennett, V (1995) The ANK repeats of erythrocyte ankyrin form two distinct but cooperative binding sites for the erythrocyte anion exchanger *J Biol Chem.* **270**, 22050–22057
5 Reid, M E, Takakuwa, Y, Conboy, J, Tchernia, G., and Mohandas, N (1990) Glycophorin C content of human erythrocyte membrane is regulated by protein 4 1. *Blood* **75**, 2229–2234
6 Sahr, K.E, Laurila, P., Kotula, L., Scarpa, A.L, Coupal, E, Leto, T L, Linnenbach, A.J., Winkelmann, J C, Speicher, D W, Marchesi, V T, Curtis, P.J., and Forget, B G (1990) The complete cDNA and polypeptide sequences of human erythroid α-spectrin. *J. Biol Chem* **265**, 4434–4443
7 Winkelmann, J.C, Chang, J G, Tse, W.T, Scarpa, A.L, Marchesi, V T, and Forget, B.G. (1990) Full-length sequence of the cDNA for human erythroid β-spectrin *J Biol Chem* **265**, 11827–11832
8 Ursitti, J.A., Kotula, L, DeSilva, T M, Curtis, P J, and Speicher, D.W (1996) Mapping the human erythrocyte β-spectrin dimer initiation site using recombinant peptides and correlation of its phasing with the α-actinin dimer site *J Biol Chem* **271**, 6636–6644
9. Ungewickell, E. and Gratzer, W. (1978) Self-association of human spectrin· A thermodynamic and kinetic study *Eur J Biochem* **88**, 379–385
10. Shahbakhti, F and Gratzer, W.B. (1986) Analysis of the self-association of human red cell spectrin *Biochemistry* **25**, 5969–5975
11 Tse, W T and Lux, S E (1999) Red blood cell membrane disorders. *Br. J. Haematol.* **104**, 2–13
12 Kitajima, H, Yamaguchi, T, and Kimoto, E. (1990) Hemolysis of human erythrocytes under hydrostatic pressure is suppressed by cross-linking of membrane proteins. *J. Biochem.* **108**, 1057–1062
13 Yamaguchi, T, Kawamura, H., Kimoto, E, and Tanaka, M (1989) Effects of temperature and pH on hemoglobin release from hydrostatic pressure-treated erythrocytes. *J. Biochem.* **106**, 1080–1085
14 Yamaguchi, T, Murata, Y, Kobayashi, J., and Kimoto, E. (1994) Effects of chemical modification of membrane thiol groups on hemolysis of human erythrocytes under hydrostatic pressure. *Biochim Biophys. Acta* **1195**, 205–210
15 Yoshino, H and Minari, O. (1987) Heat-induced dissociation of human erythrocyte spectrin dimer into monomers. *Biochim. Biophys. Acta* **905**, 100–108
16. Yamaguchi, T, Yamamoto, M, and Kimoto, E (1996) Release of spectrin-containing vesicles from human erythrocyte ghosts by dimyristoylphosphatidylcholine. *J. Biochem.* **119**, 95–99
17 Dodge, J T, Mitchell, C, and Hanahan, D J (1963) The prepa-

ration and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**, 119–130
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
20 Yamaguchi, T., Kajikawa, T., and Kimoto, E. (1991) Vesiculation induced by hydrostatic pressure in human erythrocytes. *J. Biochem.* **110**, 355–359
21 Ott, P., Hope, M.J., Verkleij, A.J., Roelofsen, B., Brodbeck, U., and Van Deenen, L.L.M. (1981) Effect of dimyristoylphosphatidylcholine on intact erythrocytes. Release of spectrin-free vesicles without ATP depletion. *Biochim. Biophys. Acta* **641**, 79–87
22. Yamaguchi, T., Yamada, S., and Kimoto, E. (1994) Effects of cross-linking of membrane proteins on vesiculation induced by dimyristoylphosphatidylcholine in human erythrocytes. *J. Biochem.* **115**, 659–663
23 Gudi, S.R.P., Kumar, A., Bhakuni, V., Gokhale, S.M., and Gupta, C.M. (1990) Membrane skeleton-bilayer interaction is not the major determinant of membrane phospholipid asymmetry in human erythrocytes. *Biochim. Biophys. Acta* **1023**, 63–72
24. Shartava, A., Monteiro, C.A., Bencsath, F.A., Schneider, K., Chait, B.T., Gussio, R., Casoria-Scott, L.A., Shah, A.K., Heuerman, C.A., and Goodman, S.R. (1995) A posttranslational modification of β-actin contributes to the slow dissociation of the spectrin-protein 4.1-actin complex of irreversibly sickled cells. *J. Cell Biol.* **128**, 805–818
25 Yamaguchi, T., Furukawa, Y., and Terada, S. (1999) Excimer fluorescence of *N*-(1-pyrenyl)iodoacetamide-labeled spectrin. *Bull. Chem. Soc. Jpn.* **72**, 2509–2513
26. Lutz, H.U., Liu, S.-C., and Palek, J. (1977) Release of spectrin-free vesicles from human erythrocytes during ATP depletion. *J. Cell Biol.* **73**, 548–560
27 Allan, D., Billah, M.M., Finean, J.B., and Michell, R.H. (1976) Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular [Ca^{2+}]. *Nature* **261**, 58–60
28 Zarkowsky, H.S. (1982) Membrane-active agents and heat-induced erythrocyte fragmentation. *Br. J. Haematol.* **50**, 361–365
29 Calvez, J.-Y., Zachowski, A., Herrmann, A., Morrot, G., and Devaux, P.F. (1988) Asymmetric distribution of phospholipids in spectrin-poor erythrocyte vesicles. *Biochemistry* **27**, 5666–5670