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ATP effects on response of human erythrocyte membrane to high pressure

Takeo Yamaguchi and Shunji Fukuzaki

Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka 814-0180, Japan

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Phosphorylation of membrane proteins in human erythrocytes is mediated by intracellular ATP levels. Such phosphorylation modulates the interactions of the bilayer with the cytoskeleton and affects the membrane stability under high pressure. In erythrocytes with high intracellular ATP levels, the bilayer-cytoskeleton interaction was weakened. Compression of such erythrocytes induced the release of large vesicles due to the suppression of fragmentation and resulted in the enhanced hemolysis. On the other hand, in ATP-depleted erythrocytes the interaction between the bilayer and the cytoskeleton was strengthened. Upon compression of these erythrocytes, the release of small vesicles due to the facilitation of vesiculation resulted in suppression of hemolysis. Taken together, these results suggest that the responses, *i.e.*, vesiculation, fragmentation, and hemolysis, of the erythrocytes to high pressure are largely modulated by the bilayer-cytoskeleton interaction, which is mediated by intracellular ATP levels.

Key words: band 3, flow cytometry, hemolysis, spectrin, vesiculation

Corresponding author: Takeo Yamaguchi, Department of Chemistry, Faculty of Science, Fukuoka University, Jonan-ku, Fukuoka 814-0180, Japan.
e-mail: takeo@fukuoka-u.ac.jp

Intracellular ATP plays an important role in the structure and functions of the erythrocyte membrane [1]. Human erythrocytes are able to synthesize 2 mole ATP from 1 mole glucose and intracellular ATP concentration is 1~2 mM [2]. Erythrocyte shape transforms from biconcave disc to echinocytic form with decreasing ATP levels [3]. In such echinocytic erythrocytes, intracellular potassium level decreases and intracellular sodium and calcium levels increase [4]. Membrane vesicles are released from such echinocytic cells and contain no spectrin, which is a major cytoskeletal protein in the erythrocyte membrane [5]. Spectrin is composed of α,β -heterodimer and exists as a tetramer in the membrane [6]. On the other hand, intracellular ATP is mainly consumed by ($\text{Na}^+\text{-K}^+$) ATPase to maintain the high level of potassium contents and the low level of sodium ones in the erythrocyte [7]. Moreover, when tissue oxygen is demanded, ATP released from the erythrocyte is utilized to increase vascular caliber so that oxygen delivery is facilitated [8,9].

Elastic properties of human erythrocytes are the membrane deformability and stability [10,11]. Normal erythrocyte with a diameter of 7~8 μm is able to pass through a capillary of about 4 μm in diameter using its deformability [2,12]. Membrane deformability and stability are mediated by the bilayer-cytoskeleton interaction that is characterized by the linkage of band 3 with spectrin *via* ankyrin [13]. Here, band 3 is a major transmembrane protein and anion exchanger in erythrocytes [10,14]. Moreover, the erythrocyte bilayer is

◀ Significance ▶

Phosphorylation and dephosphorylation of cytoskeletal proteins in the human erythrocyte membrane mediate the bilayer-cytoskeleton interaction that plays an important role in the membrane stability and deformability. However, there is few studies using whole cells on the relation between intracellular ATP levels and membrane stability. Responses, *i.e.*, vesiculation, fragmentation, and hemolysis, of erythrocytes to high pressure were greatly modulated by intracellular ATP levels. Vesiculation, particularly, size of released vesicles, was associated with the pressure-induced hemolysis. Thus, the present data provide useful information about *in vivo* vesiculation.

attached to cytoskeleton through the linkage of glycoporphin C with the actin junctional complex containing protein 4.1R (erythrocyte membrane protein band 4.1), p55 (palmitoylated membrane protein 1), and other proteins [15]. Band 3 is also connected to spectrin *via* actin junctional complex composed of protein 4.1R, protein 4.2 (erythrocyte membrane protein band 4.2), and adducin [15]. The mutation of proteins that participate in the bilayer-cytoskeleton interaction induces the abnormal protein-protein interactions [13]. Thus, the default in the bilayer-cytoskeleton interaction results in the loss of membrane deformability and stability followed by hemolysis and membrane vesiculation [13,15].

Rheological study of erythrocytes has been performed using various methods such as ektacytometer [11], pipette aspiration technique [16], high pressure [17,18], *etc.* Force applied to erythrocytes in the ektacytometer and pipette aspiration technique is anisotropic, whereas isotropic force is applied to them in the case of high pressure. Membrane stability and deformability are readily measurable using an ektacytometer [11,19]. The pipette aspiration technique is suitable to detect the integral proteins that are not linked to cytoskeletal proteins [16]. On the other hand, we have been demonstrating that the response of the erythrocyte membrane to high pressure reflects the bilayer-cytoskeleton interaction [18,20]. When human erythrocytes are exposed to high pressures for 30 min at 37°C, the hemolysis, vesiculation, and fragmentation begin to occur at 140 MPa and the value of hemolysis at 200 MPa is about 50% [18,21,22]. This value of hemolysis at 200 MPa changes sensitively upon membrane perturbation [20,22,23]. Here, vesicles and fragmented particles are produced from erythrocytes or mother cells [18,21–23]. Mother cells are mainly produced by vesiculation of erythrocytes (intact cells) [18,21–23]. Vesicles are particles with diameter below 600 nm and fragmented particles between mother cell and vesicles in size [18,21–23]. Therefore, the size of cells or particles is as follows: erythrocyte > mother cell > fragmented particle > vesicle. Interestingly, the proportion of vesiculation, fragmentation, and hemolysis is modulated by chemical modification [23] and enzymatic digestion [21,22] of erythrocyte membrane proteins. However, such treatments of membrane proteins are unphysiological modifications. So, it is important to examine the effect of physiological modifications such as phosphorylation and dephosphorylation of membrane proteins on the behavior of the erythrocyte membrane under pressure. Such physiological modifications are metabolically regulated by changing intracellular ATP levels [24]. Moreover, effects of ATP on shape and stability of the erythrocyte membrane have been intensively investigated [19,24]. Thus, it is of interest to examine the effect of intracellular ATP on the behavior of erythrocytes under high pressure. In the present work, we show that the responses of human erythrocytes to high pressure are modulated by the bilayer-cytoskeleton interaction, which is mediated by phosphorylation or dephosphorylation of cytoskeletal proteins.

Materials and Methods

Materials

Compounds were obtained from the following sources: adenosine, glucose, inosine, Wako chemicals; adenosine 5'-(β , γ imido) triphosphate (ADPNP), adenosine 5'-triphosphate magnesium salt (ATP), Sigma; N-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide hydrochloride (A3), Calbiochem.; eosin-5-maleimide, Molecular Probes; 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), Tokyo Kasei; octaethylene glycol mono-*n*-dodecyl ether (C₁₂E₈), Nikko Chemicals. All other chemicals were of reagent grade.

Regulation of intracellular ATP levels

Human erythrocytes were obtained from the Kyushu Red Cross Blood Center. The reserved erythrocytes were washed three times with phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) by centrifugation for 10 min at 1,000×*g* and 4°C. To regenerate ATP within the erythrocytes, the cells at a 20% hematocrit were incubated in PBS containing 5 mM glucose, 2 mM adenine, and 10 mM inosine for 0.5–2 h at 37°C [25]. On the other hand, the ATP-depleted erythrocytes were prepared by incubating the cells in PBS containing penicillin G (0.2 mg/mL) for 24 h at 37°C [5]. These erythrocytes were used for ghost preparation and hemolytic experiments. For the determination of ATP concentration within the erythrocytes by ³¹P-NMR, the cells were washed in 0.9% NaCl solution. The ³¹P NMR spectra were recorded at 25°C with no spinning of a 10-mm round bottom NMR tube containing both erythrocyte suspension and a small glass capillary filled with 85% H₃PO₄. The ³¹P NMR spectra run at 161.8 MHz on a JEOL JNM-GSX 400 spectrometer. For the detection of intracellular ATP by a MALDI-TOF-MS, erythrocytes (50 μ L) were suspended in 150 μ L of acetonitrile containing 0.7 mM guanosine mono-phosphate (GMP) [26]. The suspension was sonicated, mixed with 100 μ L of H₂O, sonicated, and centrifuged for 10 min at 15,000×*g* and 4°C. As MS samples, equal volumes of supernatant and 9-aminoacrydine (10 mg/mL) were mixed [27]. The MS measurement was carried out on an Autoflex speed MALDI-TOF-MS instrument with linear mode (Bruker).

ATP effects on membrane stability were confirmed using red ghosts loaded with ATP and/or other chemicals. Erythrocytes (0.1 mL) were hemolyzed with 0.2 mL of 5 mM sodium phosphate, pH 8 (5P8) containing 5 mM ATP or 5 mM ADPNP at 0°C [28]. As a kinase inhibitor, 2 μ L of 10 mM A3 in DMSO was added into the hemolyzate [29]. These hemolyzates were incubated for 10 min at 0°C. After the incubation, 10.5 μ L of resealing solution (2.8 M KCl, 0.2 M NaCl, 20 mM MgCl₂) was added to the hemolyzate. To reseal the membrane, these hemolyzates were incubated for 1 h at 37°C. These resealed red ghosts were washed three times with PBS.

Pressure-induced morphological changes and hemolysis

To examine morphological changes of erythrocytes by pressure, reserved erythrocytes at a 10% hematocrit in PBS were incubated for 0 and 24 h at 37°C and exposed to a pressure of 200 MPa for 30 min at 37°C [17]. After the decompression up to 0.1 MPa (atmospheric pressure), the suspensions were used as samples of a light microscope (model IX-71, Olympus).

Pressure-induced hemolysis was performed as previously described [17,22]. Briefly, the erythrocytes (or red ghosts) suspended at a 0.3% (or 0.6% for red ghosts) hematocrit in PBS were exposed to a pressure of 200 MPa for 30 min at 37°C. After the decompression, the erythrocyte suspensions were centrifuged for 1 min at 3,000×*g* and room temperature. The absorbance of the supernatants was measured at 542 nm. The particles produced from erythrocytes by high pressure were analyzed using an EPICS XL-MCL flow cytometer (Coulter, USA) [21,22].

Bilayer-cytoskeleton interaction

To extract the cytoskeletal proteins from the membrane, open ghosts, which were prepared from the erythrocytes using 5P8, were suspended in 3 volume of 0.1 mM EDTA (pH 8) and incubated for 12 h at 0°C. After the incubation, the ghost suspensions were centrifuged for 20 min at 20,000×*g* and 4°C. The protein concentration in the supernatant was determined by the method of Lowry *et al.* [30]. To examine band 3-cytoskeleton interactions by labeling band 3 with eosin-5-maleimide, erythrocytes (20% hematocrit) in PBS were pretreated with 0.5 mM DNDS at 37°C for 10 min and treated with eosin-5-maleimide (0.1 mg/ml) for 1 h at room temperature [22]. After the incubation, the erythrocytes were washed three times with PBS, twice with PBS containing 0.5% (w/v) bovine serum albumin, and then three times with PBS. Red ghosts prepared from eosin-5-maleimide-labeled erythrocytes using 2 volume of 5P8 containing 5 mM ATP were incubated for 10 min at 0°C. These hemolyzates made isotonic by adding the resealing solution were incubated at 37°C for 1 h. These resealed red ghosts were washed with PBS and used to prepare white ghosts by 5P8. Here, white ghosts were prepared to exclude the influence of hemoglobin on the fluorescence intensity of eosin. White ghosts were solubilized by 0.5% (w/v) C₁₂E₈ in 5P8 for 10 min at 0°C. Thus, solubilized samples were centrifuged at 20,000×*g* for 20 min at 4°C. The proportion of band 3 interacted with cytoskeleton was estimated from fluorescence intensities of eosin-5-maleimide in supernatants and pellets. The fluorescence of eosin was measured with excitation at 490 nm and emission at 546 nm using a model FP-750 spectrometer (JASCO).

Vesicle properties

Reserved erythrocytes in glucose-free PBS at a 10% hematocrit were preincubated for 0 or 24 h at 37°C and washed with PBS. The preincubated erythrocytes were

exposed to a pressure of 200 MPa for 30 min at 37°C. For ATP-depleted vesicles (A-vesicles), reserved erythrocytes in glucose-free buffer at a 10% hematocrit were incubated for 24 h at 37°C and 0.1 MPa. These erythrocyte suspensions were centrifuged for 10 min at 1,000×*g* and room temperature. The supernatants were filtered through a Millipore filter of pore size 3.0 μm. The filtrates were centrifuged for 20 min at 20,000×*g* and 4°C. Here, the pellets prepared from the incubation for 24 h at 37°C and 0.1 MPa, or prepared from the compression of erythrocytes preincubated for 0 or 24 h at 37°C were referred to as A-, P- or AP-vesicles, respectively. These vesicles were suspended in PBS and used to measure particle size by dynamic light scattering using a Zetasizer Nano S (Malvern Instruments). Membrane proteins in vesicles and ghosts were separated by SDS-PAGE using 8% acrylamide, according to the method of Laemmli [31]. The gels were stained for proteins with Coomassie Brilliant Blue.

Results

Pressure-induced hemolysis of human erythrocytes is enhanced by increment of intracellular ATP levels

Intracellular ATP levels in human erythrocytes were determined using ³¹P-NMR (Fig. 1A) [32,33]. To increase intracellular ATP levels, reserved erythrocytes were suspended in ATP-regenerating buffer and incubated for 0.5–2 h at 37°C. At the maximum level, ATP concentration increased 2.5-fold, compared with that of reserved erythrocytes. When these erythrocytes were exposed to a pressure of 200 MPa for 30 min at 37°C, pressure-induced hemolysis was enhanced upon increment of intracellular ATP levels (Fig. 1B). The responses of the erythrocytes to a pressure of 200 MPa were analyzed by the flow cytometry (Fig. 1C). Forward scatter *versus* side scatter dot plots were divided into four regions (**a**, **b**, **c**, and **d**), taking both scatter histograms into consideration. As described previously, regions **a**, **b**, **c**, and **d** contain mother cells, fragmented particles, vesicles, and open ghosts, respectively [21–23]. ATP-regenerated erythrocytes were refractory to the fragmentation under pressure so that the hemolysis was readily occurred, as indicated in the increase of open ghosts (Fig. 1C).

Hemolytic properties at 200 MPa of resealed red ghosts containing ATP, kinase inhibitor, or ADPNP

β-Spectrin is readily phosphorylated by intracellular ATP [19,24]. So, we examined the effect of intracellular ATP on pressure-induced hemolysis using resealed red ghosts (Fig. 2A). Pressure-induced hemolysis was enhanced by ATP loading within resealed red ghosts. However, such enhancement was suppressed by A3, kinase inhibitor. Moreover, ADPNP is a nonhydrolyzable analog of ATP [28]. When ADPNP was loaded instead of ATP, no enhancement of hemolysis at 200 MPa was observed. These results suggest that the phosphorylation of erythrocyte membrane proteins is associated with the enhancement of pressure-induced

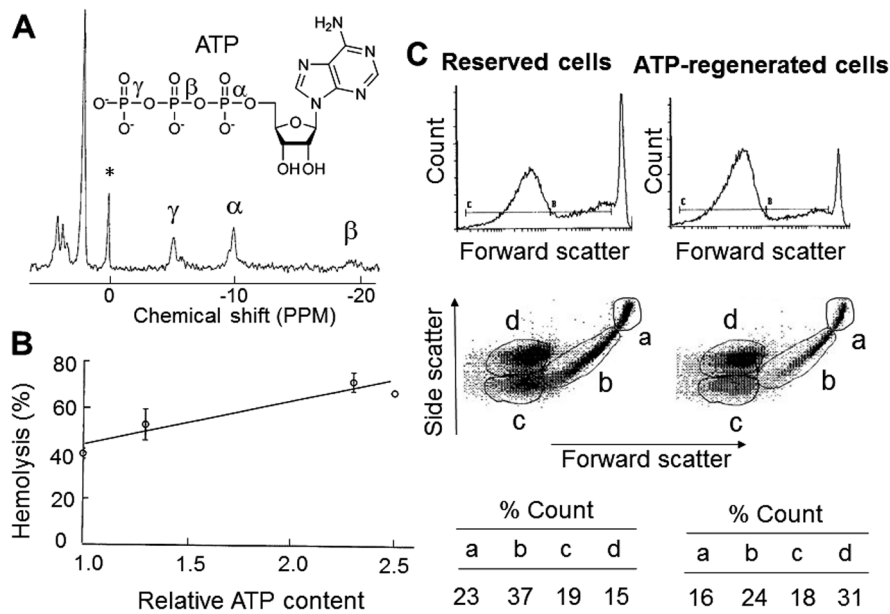


Figure 1 Effects of intracellular ATP levels on the responses of human erythrocytes to a pressure of 200 MPa. (A) ^{31}P -NMR spectrum of reserved erythrocytes. *external reference of 85% H_3PO_4 . (B) Relationship between pressure-induced hemolysis and intracellular ATP contents in erythrocytes. Values are means \pm SD for at least two independent experiments. (C) Flow cytometric analysis of reserved erythrocytes and ATP-regenerated ones exposed to a pressure of 200 MPa. For each measurement, 50,000 particles were counted.

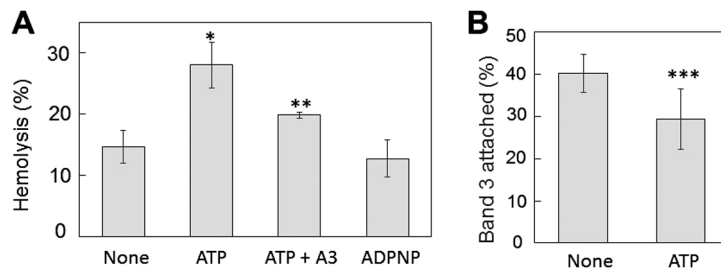


Figure 2 Effects of intracellular ATP on pressure-induced hemolysis and band 3-cytoskeleton interaction in red ghosts. (A) Hemolytic properties of red ghosts exposed to a pressure of 200 MPa. Reserved erythrocytes hemolyzed in 5P8 containing 5 mM ATP, 5 mM ADPNP, or 0.1 mM A3 were resealed under isotonic conditions. (B) Band 3-cytoskeleton interaction. Band 3 in reserved erythrocytes was labeled with eosin. White ghosts prepared from ATP-loaded red ghosts were used to examine the interaction. Values are means \pm SD for three independent experiments. * $p < 0.01$ vs. none; ** $p < 0.03$ vs. ATP; *** $p < 0.02$ vs. none

hemolysis. Pressure-induced hemolysis is affected by the bilayer-cytoskeleton interaction [21,22]. So, the interaction of band 3 with cytoskeletal proteins was examined using C_{12}E_8 , nonionic detergent (Fig. 2B). In this method, when ghosts are solubilized by C_{12}E_8 , band 3 that is attached to cytoskeleton is observed with cytoskeletal proteins as pellets by centrifugation [22]. Upon ATP loading, the amount of band 3 contained in the pellet was significantly decreased (Fig. 2B). This indicates a weak interaction of band 3 with cytoskeleton, which is induced by the phosphorylation of membrane proteins [34].

Compression of ATP-depleted erythrocytes facilitates the vesiculation so that pressure-induced hemolysis is suppressed

To examine the effect of intracellular ATP levels on the membrane stability of erythrocytes under pressure, it is useful to use ATP-depleted cells. It is well known that the intracellular ATP levels are decreased during the incubation of erythrocytes at 37°C in glucose-free buffer [5]. The morphology of the reserved erythrocytes was echinocytic in most cells due to the decrease of intracellular ATP (Fig. 3A-①) [3]. When such reserved erythrocytes were exposed to a pressure of 200 MPa for 30 min at 37°C and decompressed up to 0.1 MPa, various sizes of fragmented particles were observed (Fig. 3A-②). On the other hand, when the reserved erythrocytes were incubated for 24 h at 37°C in

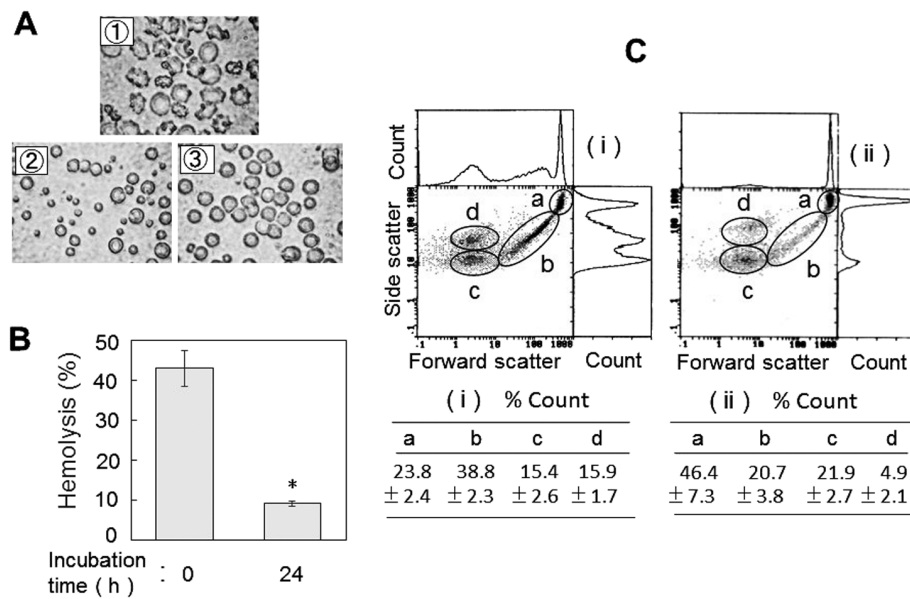


Figure 3 Pressure response of erythrocytes preincubated for 24 h at 37°C. Reserved erythrocytes were incubated for 0 and 24 h at 37°C in glucose-free buffer and exposed to a pressure of 200 MPa. (A) Light microscopy at 0.1 MPa of erythrocyte suspensions. ①, cells at 0.1 MPa without incubation (control); ② and ③, cells compressed after incubation for 0 and 24 h at 37°C, respectively. (B) Pressure-induced hemolysis of erythrocytes incubated for 0 and 24 h at 37°C. Values are means±SD for three independent experiments. **p*<0.01 vs. 0 h. (C) Flow cytometric analysis of 200 MPa-treated erythrocyte suspensions. Erythrocytes were incubated for 0 (i) or 24 h (ii) at 37°C prior to compression. For each measurement, 20,000 particles were counted. Values are means±SD for three independent experiments.

glucose-free buffer and exposed to a high pressure, most cells were spherical and large size (here, these cells are referred to as mother cells) (Fig. 3A-③). Moreover, the pressure-induced hemolysis also was suppressed greatly upon incubation for 24 h at 37°C (Fig. 3B). To analyze quantitatively the size distribution of particles produced from pressure-treated erythrocytes, we measured the flow cytometry. Upon compression of 24 h-incubated erythrocytes, the fragmentation was suppressed but vesiculation was facilitated. In this case, the hemolysis was greatly suppressed (Fig. 3C). Here, the region **a** contains many large particles (or mother cells) which their size distribution is relatively sharp. Thus, the response of reserved erythrocytes to high pressure changed dramatically upon incubation for 24 h at 37°C in glucose-free buffer. To confirm the decrease of ATP level during incubation at 37°C, the intracellular ATP concentration was examined using a MALDI-TOF-MS. Upon incubation for 24 h at 37°C in glucose-free buffer, the intracellular ATP was completely depleted (Fig. 4A). The bilayer-cytoskeleton interaction plays an important role in the stability and deformability of the erythrocyte membrane. Cytoskeleton such as spectrin and actin is extracted from the membrane under hypotonic conditions [20,23]. The amount of extracted proteins was significantly decreased in 24 h-incubated erythrocytes, compared with no-incubated ones (Fig. 4B). These results suggest that the interaction of the bilayer with cytoskeleton is strengthened upon depletion of intracellular ATP and such strong interaction facilitates the vesiculation of erythrocytes under pressure.

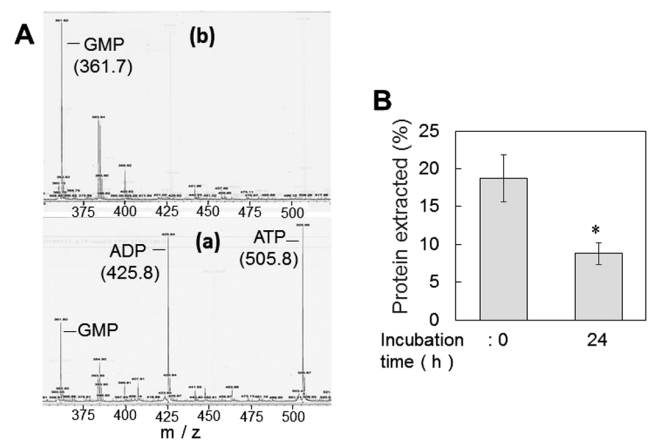


Figure 4 Intracellular ATP level and bilayer-cytoskeleton interaction in reserved erythrocytes incubated without glucose for 24 h at 37°C. (A) MS spectra of intracellular ATP in erythrocytes incubated for 0 (a) and 24 h (b) at 37°C. GMP was used as an internal reference. (B) Extraction of cytoskeleton from the ghost membrane. Ghost membranes were prepared from erythrocytes incubated for 0 and 24 h at 37°C. Values are means±SD for three independent experiments. **p*<0.05 vs. 0 h

Properties of vesicles released from ATP-depleted erythrocytes by pressure

When human erythrocytes were exposed to a pressure of 200 MPa, membrane vesicles were released from the cell surface (P-vesicles) [18]. On the other hand, vesicles were also released from the surface of ATP-depleted erythrocytes

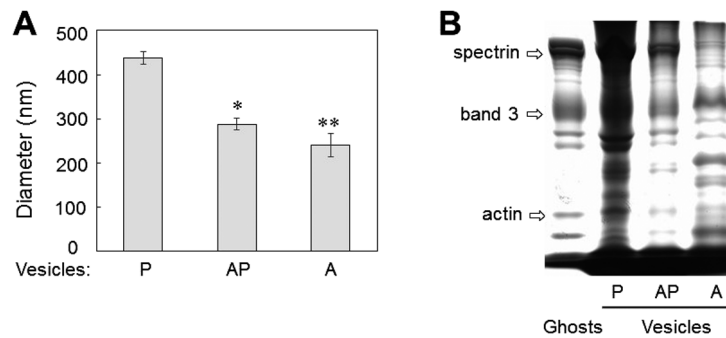


Figure 5 Size and protein composition of pressure-induced vesicles and ATP depletion-induced ones. P-vesicles and AP-ones were prepared by compression under 200 MPa of erythrocytes preincubated for 0 and 24 h without glucose at 0.1 MPa and 37°C, respectively. A-vesicles were prepared by incubation for 24 h without glucose at 0.1 MPa and 37°C. (A) Determination of vesicle size by light scattering. Values are means±SD for three independent experiments. *** $p < 0.01$ vs. P-vesicles. (B) SDS-PAGE of erythrocyte membrane proteins in ghosts and vesicles.

that were prepared by long incubation at 37°C in glucose-free buffer (A-vesicles) [5]. Moreover, upon compression of ATP-depleted erythrocytes the vesiculation was facilitated. Thus released vesicles are referred to as AP-vesicles. The size of these vesicles was determined by a light scattering method. Diameters of P-, AP-, and A-vesicles were 437.8 ± 14.9 , 287.8 ± 13.4 , and 240.8 ± 26.1 nm ($n=3$), respectively (Fig. 5A). Moreover, we examined the membrane protein compositions in these vesicles using SDS-PAGE (Fig. 5B). As shown previously [5], the content of spectrin was poor in A-vesicles. On the other hand, the spectrin content clearly increased in P- and AP-vesicles, compared with that of A-vesicles.

Discussion

Phosphorylation and dephosphorylation of membrane proteins in human erythrocytes are mediated by intracellular ATP levels [35]. It is well known that β -spectrin is phosphorylated under high intracellular ATP levels [19,24]. Moreover, phosphorylation of protein 4.1R is induced by protein kinase C in the presence of phosphatase inhibitors [36]. On the other hand, ATP levels within erythrocytes decrease upon long incubation of them in glucose-free buffer at 37°C so that dephosphorylation of membrane proteins is induced [5,37,38]. Thus, the interaction of the bilayer with cytoskeleton is reduced by phosphorylations of β -spectrin [19], protein 4.1R [36], ankyrin [35], and band 3 [34], whereas is enhanced by dephosphorylation of such proteins [37,38].

Mechanical deformability and stability of human erythrocytes are mediated by the bilayer-cytoskeleton interactions [34,36–38]. For instance, anion exchanger band 3 is associated with spectrin *via* ankyrin complex or actin junctional complex [34]. Moreover, glycophorin C that is a transmembrane protein is also linked to spectrin *via* actin junctional complex [15]. Therefore, the defaults in the vertical interaction of transmembrane proteins with cytoskeleton by mutation of these proteins result in spherocytosis due to vesiculation [13]. Furthermore, the interactions of bilayer

with cytoskeleton are also decreased upon phosphorylation of spectrin or proteins 4.1R so that the erythrocyte membranes are fragmented under shear stress, which is anisotropically applied, as demonstrated by the ektacytometer [19,36]. On the other hand, the behavior under high pressure of such phosphorylated erythrocytes is different from that under the ektacytometer. In the case of high pressure, the applied force is isotropic for the erythrocytes suspended in buffer. When the ATP-rich erythrocytes are exposed to high pressure, the cell shape becomes spherical and the pressure-induced hemolysis is enhanced. Similar results are observed in the erythrocytes incubated in alkaline pH buffer that reduces the interaction of bilayer with cytoskeleton [39,40]. On the contrary, the connection of the bilayer with the cytoskeleton increases in the ATP-depleted erythrocyte, as shown in the present work [41]. The pressure-induced hemolysis of such erythrocytes is greatly suppressed.

Here, it is useful to consider the relationship among hemolysis, vesiculation, and fragmentation of erythrocytes induced by high pressure (Fig. 6). When human erythrocytes are exposed to a pressure of 200 MPa for 30 min at 37°C, hemolysis of about 50%, vesiculation, and fragmentation are observed [17]. As a typical example, we consider the case of 49°C-treated erythrocytes, where spectrin is denatured [41]. This denatured spectrin is tightly associated with the bilayer and membrane vesiculation begins to occur with a little hemolysis [41,42]. Thus released vesicles at 49°C are spectrin-poor and a diameter about 390 nm in size [43]. Upon compression at 37°C of 49°C-pretreated erythrocytes, the vesiculation is predominantly facilitated and the hemolysis and fragmentation are greatly suppressed, as with Figure 3 [21]. Here, released vesicles are about 260 nm in size and contain small amount of spectrin [43]. On the other hand, the hemolysis is largely enhanced when the erythrocytes pretreated with proteases such as trypsin [21,44] and papain [22] or sodium *p*-chloromercuribenzoate (*p*CMB)-pretreated ones [23] are exposed to high pressure. In this case, although the fragmentation is remarkably suppressed, the size of released vesicles is large, *i.e.*, 574, 525, or 580 nm for each

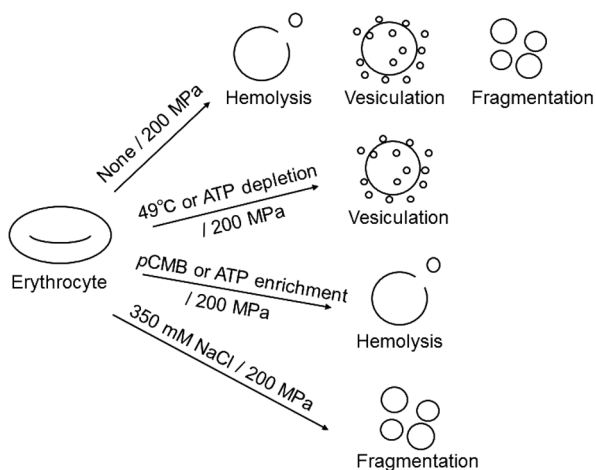


Figure 6 Model for response of human erythrocytes to high pressure. Intact erythrocytes and chemically or enzymatically modified ones in isotonic or hypertonic buffer were exposed to a pressure of 200 MPa. Major events induced by pressure are depicted.

case of trypsin [21], papain [22] or *p*CMB [23], respectively. Moreover, the facilitation of pressure-induced fragmentation also suppresses the hemolysis and vesiculation, as seen in erythrocytes exposed to high pressure under hypertonic conditions (10 mM sodium phosphate, 350 mM NaCl, pH 7.4) (Supplementary Fig. S1). These data suggest that the facilitation of the vesiculation characterized by the release of small vesicles and the fragmentation suppresses the pressure-induced hemolysis. Taking these facts into account, we explain the intracellular ATP effects on the response of the erythrocyte membrane to applied pressure. In normal erythrocytes, the cytoskeleton is linked to the bilayer at defined sites [13,15]. Therefore, the morphological and mechanical properties of the bilayer are severely controlled by the cytoskeleton [19,38]. In ATP-rich erythrocytes or ATP-loaded red ghosts, spectrin is phosphorylated so that the interaction of cytoskeleton with the bilayer reduces [24,36]. Thus, the bilayer is considerably relaxed from the restriction by the cytoskeleton. When such erythrocytes are exposed to a high pressure, the suppression of the fragmentation and/or vesiculation induced by pressure results in enhancement of hemolysis characterized by the release of large size of vesicles. On the other hand, the interaction of cytoskeleton with the bilayer is strengthened upon depletion of ATP so that the surface tension of the bilayer increases [37]. Microscopically, the cytoskeletal spectrin pulls the inner leaflet of the bilayer so that the outer leaflet expands relative to the inner one [3,37]. According to the bilayer couple hypothesis by Sheet and Singer [45], the morphology of erythrocytes changes into echinocytic form under conditions of ATP depletion [3,24,46]. Membrane vesicles released from such echinocytic erythrocytes are about 180–240 nm in size and spectrin-free [5]. When such erythrocytes are exposed to a high pressure, the vesiculation characterized by small size of

vesicles is facilitated so that the hemolysis and fragmentation are greatly inhibited. In these small vesicles, spectrin content is decreased, compared with that in ghosts.

Conclusion

In vivo, the membrane vesicles are produced from circulating senescent erythrocytes [47]. Various sizes of vesicles are contained in the plasma [47]. Judging from the properties of vesicles produced by ATP depletion [5], heating [42,43], and pressure [18,24], the *in vivo* vesicles below 300 nm in diameter also seems to be spectrin-free or -poor. The present work suggest that the *in vivo* vesiculation of senescent erythrocytes is followed by no hemolysis in the case of the release of small vesicle below 200 nm in diameter. Moreover, the responses, *i.e.*, hemolysis, vesiculation, and fragmentation, of human erythrocytes to high pressure are significantly modulated by the intracellular ATP levels that mediate the bilayer-cytoskeleton interaction through the phosphorylation or dephosphorylation of membrane proteins. Particularly, the abnormality of the bilayer-cytoskeleton interaction that is mediated by the intracellular ATP is sensitively reflected to the value of pressure-induced hemolysis. Interestingly, erythrocyte flickering (or fluctuation) is characterized by slow process at long timescales above 100 ms and rapid one at short timescales at below 100 ms [48]. Slow fluctuations are observed in ATP-rich erythrocytes, whereas rapid ones in ATP-depleted cells. Thermal agitation mainly contributes to the rapid fluctuations [48]. Thus, slow fluctuations are suppressed by ATP depletion. Further study is necessary whether erythrocyte flickering is associated with the response of the erythrocyte membrane to high pressure or not.

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Conflicts of interest

T. Y. and S. F. declare that they have no conflict of interest.

Author contribution

T. Y. directed the entire project and wrote the manuscript. T. Y. and S. F. performed all experiments.

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