Marked Impairment of Human Erythrocyte Filterability Caused by Oxidant Stress with AAPH Precedes Oxidative Hemolysis

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Erythrocytes as oxygen carriers are inevitably exposed to persistent oxidant stress, which determines in part the life span of circulating erythrocytes. Therefore, hemolytic and hemorheological effects of oxidant stress caused by 50 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) on intact human erythrocytes obtained from healthy volunteers (n = 6, mean age of 28.9 ± 10.3 years) were investigated. Incubation of erythrocyte suspension (3% in hematocrit) with 50 mM AAPH at 36°C up to 3 hours increased the mean corpuscular volume of erythrocytes (from 83.0 ± 2.0 to 113.7 ± 2.5 fl finally, p < 0.001), and caused small but significant (p < 0.001) increases of methemoglobin (metHb) formation (5.8 ± 0.8%) and hemolysis to a final extent of 16.9 ± 2.8% in a time-dependent manner. Erythrocyte filterability (whole-cell deformability) was assessed by a highly sensitive and reproducible nickel mesh filtration technique, and defined as flow rate of erythrocyte suspension relative to that of saline (%) using the pressure–flow rate curve at a filtration pressure of 100 mmHg. Treatment with AAPH impaired the erythrocyte filterability in a time-dependent sigmoidal manner from 82.0 ± 1.2% to 12.0 ± 2.9%, which was pronounced 40 to 60 minutes after starting incubation. On the other hand, accelerated hemolysis was observed at least 60 minutes after starting exposure. These hematological and hemorheological findings indicate that oxidant stress caused by AAPH demonstrates time-dependent impairment of human erythrocyte filterability, erythrocyte swelling, metHb formation associated with chain-reacting erythrocyte membrane damage and resultant oxidative hemolysis. By simultaneous observations after starting oxidative stress, hemorheological impairment of erythrocyte behavior preceded the oxidative hemolysis. In this sense, erythrocyte filterability (whole-cell deformability), a prerequisite of microcirculation in vivo, is concluded to be a sensitive and clinically relevant parameter of oxidative erythrocyte damage leading to consequent hemolysis.

Key words: AAPH/erythrocytes/filterability/membrane/nickel mesh/oxidative stress

1. Introduction

Although reactive oxygen species (ROS), byproducts of biological oxygen consumption, correspond to only 0.1 to 0.2% of utilized oxygen, ROS play a key role in oxidative stress 1). Oxidative stress involves various chronic health problems such as diabetes, cancer, senescence, and many circulatory disorders including atherosclerosis, hypertension, stroke and myocardial infarction. Microcirculation in vivo is also impaired by oxidative stress caused by ROS 2). However, the mechanisms of this impairment are complicated at the cellular membrane, protein, lipid, and genome levels. Oxidative stress has a profound hemorheological impact on circulating erythrocytes. Erythrocytes are a major cellular component of circulating blood and a physiological oxygen carrier. Erythrocytes are also

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sensitive to oxidant stress, because they are exposed to the redox (oxygenation/deoxygenation) cycle under intact in vivo circulation in spite of their intrinsic antioxidant defense mechanisms.

To date, many hemorheological methodologies have been established. However, these are diverse with different levels of sensitivity, specificity and reproducibility5. Moreover, the link between hemorheological and hematological derangements of erythrocytes exposed to an oxidative environment has not been fully investigated. Therefore, we assessed the hemorheological and hematological effects of oxidant stress caused by 2,2’-azobis(2-aminopropane) dihydrochloride (AAPH) on human erythrocytes obtained from healthy volunteers, because AAPH is a small–molecular hydrophilic free radical initiator generating molecular nitrogen and 2 carbon radicals. This agent is currently used for investigations of biological lipid peroxidation and antioxidative potency of endogenous substances and exogenous food4, 5–7).

2. Experimental

2.1 Subjects

This study design was performed according to the Declaration of Helsinki (2000) and approved by the internal ethics committee of The Institute of Rheological Function of Foods Co. Ltd. (Hisayama, Fukuoka, Japan), with signed informed consent being obtained from subjects prior to enrollment in the study. Approximately 15 ml of venous blood was drawn from the antecubital vein of apparently healthy volunteers (n = 6, mean age of 28.9 ± 10.3 years) using 21–gauge needles and disposable evacuated syringes (Terumo Associates, MA, USA). Erythrocytes were then washed three times at 4 °C by repeated re-suspension with HEPES–NaOH–buffered saline and centrifugation at 800 × g, 600 × g and 500 × g for 10 minutes each. The final hematocrit of human erythrocyte suspension was adjusted to 3.0%. These procedures were performed within 2 hours after blood sampling for subsequent experiments.

AAPH (C₉H₁₈N₆·2HCl; MW = 271.2), a water–soluble radical generator of azo compound, was commercially obtained from Wako Pure Chem., Co. Ltd. (Osaka, Japan) and stored in a refrigerator at −20 °C. The total volume of the reaction mixture was brought up to 5 ml by adding HEPES–NaOH–buffered saline. After preincubation at 36 °C for 5 minutes, the reaction was started by adding AAPH at a final concentration of 50 mM as in our previous study9). During this exposure, the mixture was incubated at 36 °C for the desired time period up to 3 hours. AAPH is so stable that the half-life of this agent is about 175 hours at 36 °C and at neutral pH, and that the rate of free radical generation is constant (rate of AAPH decomposition of 1.3 × 10⁻⁶ [AAPH]/sec) during the first several hours in reaction mixture10). Therefore, the reaction was terminated by cooling the test tubes in an ice bath, and the erythrocytes were pelleted by centrifugation at 1000 × g for 5 minutes at 4 °C. Thereafter, AAPH–treated erythrocytes were washed twice and re-suspended with HEPES–NaOH–buffered saline.

2.3 Erythrocyte volume estimation

Blood cell counting and hematocrit measurements were carried out using a hemocytometer (Ace Counter, FLC–240A, Fukuda Denshi Co. Ltd., Tokyo, Japan). Mean corpuscular volume of erythrocytes (MCV; fl) was calculated automatically using this hemocytometer as a surrogate of average erythrocyte volume. After starting incubation with AAPH (50 mM) at 36 °C, MCV was evaluated using re-suspension of AAPH–treated erythrocytes up to 3 hours after starting exposure. MCV estimation was performed at room temperature (22 ± 3 °C). This parameter was also estimated in an erythrocyte suspension left for 3 hours without an exposure to AAPH.
2.4 Methemoglobin assay

An aliquot of the reaction mixture (0.4 ml) was hemolyzed using 5 ml of solution containing 100 mM phosphate buffer and 1% Triton X-100 (4:6 in volume ratio, pH 6.8) and then hemolysate was divided into two parts. The absorption peak at 630 nm of one part was read in the absence and presence of 5% potassium cyanide. The absorption peak of another part was read at 630 nm in the presence of 5% potassium ferricyanide and then read again at 630 nm after the addition of 5% potassium cyanide. The formation of methemoglobin (met-Hb, %) was calculated as previously reported\(^1\). Measurements were performed sequentially after starting exposure to 50 mM AAPH at 36°C, and these measurements were conducted at room temperature (22 ± 3°C).

2.5 Erythrocyte hemolysis assay

Erythrocyte suspension was subjected to exposure to 50 mM AAPH with different incubation times up to 3 hours. The reaction mixture was shaken gently at 36°C. At the desired interval, a small sample (400 μl) of reaction mixture was removed and diluted with 8 ml of HEPES–NaOH–buffered saline and centrifuged at 1000 × g for 5 minutes. Hemolysis was determined by the absorbance of hemoglobin at 540 nm in this supernatant\(^2\)–\(^4\). Percent hemolysis (%) before and after starting incubation with AAPH was compared with complete hemolysis by treating the same erythrocyte suspension with distilled water. Hemolysis assay was performed at room temperature (22 ± 3°C).

2.6 Erythrocyte filterability measurement

Hemorheological equipment investigating erythrocyte filterability was introduced elsewhere\(^5\)–\(^8\). A nickel mesh filter was produced in accordance with our specifications by a photofabrication technique (Dainippon Printing Co. Ltd., Tokyo, Japan). We specified that this filter should have an outer diameter of 13 mm, a filtration area 8 mm in diameter, 11 μm thickness and with an interpore distance of 35 μm (Tsukasa Sokken Co. Ltd., Tokyo, Japan). The vertical and cylindrical pores were distributed regularly across the filter without coincidence or branching. The pore entrances exhibited round and smooth transition into the pore interior. Pore diameters were all exactly identical in a specific nickel mesh filter. Filters with a specific pore diameter ranging from 3 to 6 μm were available to be selected depending on the suspension materials. After repeating the preliminary experiments to choose an appropriate pore size, a nickel mesh filter with a pore diameter of 5.31 μm was chosen for human erythrocyte suspensions.

Filtration experiments were performed blindly using a gravity–based nickel mesh filtration apparatus (Model NOBU–II, Tsukasa Sokken Co. Ltd., Tokyo, Japan). In brief, the relationship between hydrostatic pressure (P; mmH\(_2\)O) and time (t; sec) was obtained during continuous filtration by gravity using a pressure transducer. P was transformed to a height of the meniscus in a vertical tube (h; mm). The tangent of the h–t curve determined by drawing points corresponding to different heights gives the rate of fall of the meniscus (dh/dt). Thereafter, by multiplying the rate of fall by the internal cross-sectional area of the vertical tube, the complete set of flow rates (Q; ml/minutes) and corresponding P, the P–Q relationship, was obtained\(^15\), \(^16\). This procedure was automatically performed by measurement software installed in a personal computer (DELL Latitude CS, Dell Inc., Round Rock, TX, USA) and monitored on the main window of the computer screen. Together with the start of data acquisition, the measurement software displays the h–t curve continuously during the filtration process. When filtration has been completed, the software displays the relationship of pressure and flow rate (P–Q curve). The h–t and P–Q curves are shown on the computer screen and stored simultaneously on Microsoft Office Excel 2003 in Windows XP (Microsoft, Tokyo, Japan). The temperature of the specimens was kept at 25°C by circulating isothermal water through a water jacket surrounding the vertical tube. The percentage of the flow rate of erythrocyte suspension to that of HEPES–NaOH–buffered saline at 100 mm H\(_2\)O was used as an index of erythrocyte filterability. Filterability measurements were performed sequentially at the desired time after starting exposure to AAPH, and these measurements were conducted at room temperature (22 ± 3°C).

2.7 Data analyses

All data are expressed as means ± SD. Normality of the distribution of the hematological and hemorheological data was assessed by Kolmogorov–Smirnov test, and the demonstrated data were normally distributed.
Therefore, time–dependent comparisons of these continuous variables were performed using the analysis of variance (ANOVA). Practical computation was performed using PASW (Predictive Analytics Software) on Windows version for Statistical Package of Social Science® (SPSS Inc., Chicago, Ill., USA). Differences with a two–sided p < 0.050 were considered significant.

3. Results

3.1 Erythrocyte volume estimation

Fig. 1 shows MCV as a function of time of an exposure to 50 mM AAPH (n = 6). Erythrocytes subjected to exposure to AAPH showed an increase in MCV, which was evident 60 minutes after the exposure (●). Erythrocytes without exposure to AAPH showed no changes in MCV (○). Symbols and bars indicate means ± SD. MCV, the mean corpuscular volume of erythrocytes (fl).

Fig. 1 Time–dependent changes of the mean corpuscular volume of erythrocytes (fl) with and without exposure to 50 mM AAPH (n = 6). Erythrocytes subjected to exposure to AAPH showed an increase in MCV, which was evident 60 minutes after the exposure (●). Erythrocytes without exposure to AAPH showed no changes in MCV (○). Symbols and bars indicate means ± SD. MCV, the mean corpuscular volume of erythrocytes (fl).

3.2 Methemoglobin assay

The AAPH–treated erythrocyte suspension exhibited a slightly dark brownish color in a time–dependent manner. Spectrophotometric analysis demonstrated the formation of met–Hb with an absorption peak at 630 nm. After 3 hours of treatment with AAPH, this agent revealed a small but significant (p < 0.001) increase of met–Hb ranging from 5.0 to 6.5% (n = 6, 5.8 ± 0.8%, means ± SD), whereas the met–Hb formation at preincubation was not detectable at all (Fig. 2). This increase of met–Hb of erythrocyte suspension at the end of exposure was also significant (n = 6, p < 0.001) compared with met–Hb levels left for 3 hours without AAPH treatment (1.2 ± 0.5%, means ± SD), indicating AAPH–triggered auto–oxidation of oxyhemoglobin.

3.3 Erythrocyte hemolysis assay

Fig. 3 shows a hemolytic time course obtained by an exposure of erythrocyte suspension to 50 mM AAPH. Incubation of erythrocytes with AAPH yielded hemolysis in a time–dependent manner, that is progression of hemolysis ranging from 6.3 to 9.8% (n = 6, 7.9 ± 1.8%, means ± SD) was observed 90 minutes after starting incubation, and 14.3 to 19.9% (n = 6, 16.9 ± 2.8%, means ± SD) hemolysis at 3 hours of exposure. Erythrocyte suspension left for 3 hours without expo-
Fig. 3  Hemolytic time course of erythrocyte suspension (\%) exposed to 50 mM AAPH (n = 6). Erythrocytes subjected to exposure to AAPH showed time-dependent hemolysis, which was evident 60 minutes after starting the exposure (●). Erythrocyte suspension without exposure to AAPH showed no evident hemolysis (○). Symbols and bars indicate means ± SD.

Fig. 4  Representative relationships between filtration pressure (P; mmH₂O) and flow rate (Q; ml/minutes) during continuous filtration experiment using HEPES–NaOH–buffered control saline and human erythrocyte suspensions obtained from healthy volunteers (n = 6). The P–Q relationships correspond to two control saline passages, passages of erythrocyte suspensions in preincubation and under incubation with 50 mM AAPH at incubation times of 20, 40, 60, 120 and 180 minutes.

Fig. 5  Summarized data of continuous filtration experiments (n = 6). Filterability of human erythrocyte suspensions exposed to 50 mM AAPH showed time-dependent reduction, which was marked at incubation times of 40 to 60 minutes after starting exposure (●), whereas the filterability of erythrocyte suspension without exposure to AAPH remained at the preincubation levels (○). Symbols and bars indicate means ± SD.

certain to AAPH displayed no evident hemolysis at all.

3.4 Erythrocyte filterability measurement

Fig. 4 indicates the results of representative filtration experiments. The pressure vs. flow rate relationship of control HEPES–NaOH–buffered saline shows a straight line passing through the origin, indicating that saline is Newtonian fluid. Filtration of control saline displayed a completely superimposable pressure–flow line, indicating that this filtration system satisfies the reproducibility. Suspension of erythrocytes obtained from different volunteers prior to an exposure to AAPH produced a concave curve showing that the erythrocyte suspension per se is originally not Newtonian. Moreover, pressure–flow curves of the control erythrocyte suspension were considerably superimposable, implying that the normal range of erythrocyte filterability estimated by this technique is quite limited. Treatment with AAPH (50 mM) decreased the flow rate of erythrocyte suspension at any given filtration pressure. These phenomena were evident in a time–dependent manner, that is, the flow–pressure curves shifted gradually to the right according to the progression of exposure to AAPH. Erythrocyte filterability was determined as flow rate of erythrocyte suspension relative to that of saline at 100 mmH₂O (%).

Fig. 5 shows the summarized data of filterability of erythrocyte suspension before and after the treatment with AAPH. Average erythrocyte filterability prior to the treatment was 82.0 ± 1.2% (means ± SD), and the final filterability 3 hours after starting the treatment was 12.0 ± 2.9% (means ± SD). This agent therefore
impaired the erythrocyte filterability progressively in a time–dependent sigmoidal manner (n = 6, p < 0.001). The impairment of erythrocyte filterability was marked during incubation times ranging from 40 minutes to 60 minutes after starting exposure to AAPH. The filterability of the erythrocyte suspension 3 hours after the preparation without an exposure to AAPH was equivalent to that of preincubation (80.1 ± 1.6%, means ± SD).

4. Discussion

Circulating erythrocytes as an oxygen carrier exposed to periodic high oxygen pressure are a main cellular target of in vivo oxidative stress. Therefore, it is of interest to assess hemorheological and hematological correlations of intact human erythrocytes exposed to AAPH, which is used widely as an oxidative stress model at the cellular level. In our laboratory, the biochemical effects of oxidative injury caused by AAPH (50 mM) on the isolated human erythrocyte membrane have been investigated. Hemolysis is the final event in the disposal of damaged erythrocytes with impaired deformability, and the mechanisms of hemolysis in AAPH–exposed erythrocytes are not fully understood. Therefore, this study was performed in this oxidant stress model using our hemorheological method.

The filterability of erythrocytes that pass through the microvascular network is an essential factor affecting physiological microcirculation. Since in vivo erythrocyte deformation involves bending, a filtration technique is a promising tool of hemorheology in that erythrocyte filterability is considered as whole–cell bending deformability. The evaluation of filterability strictly depends on the measurement technique, and the nickel mesh filtration technique is highly sensitive, quantitative and reproducible by assessing the physiological bending deformation of intact erythrocytes. As a matter of fact, the present study demonstrated clearly that the normal filterability of human erythrocytes was quite limited to pretty narrow range around 80%, the filterability of erythrocytes exposed to 50 mM AAPH is dramatically impaired, and this impairment is accelerated 40 to 60 minutes after starting incubation (Fig. 5). By incubation for 3 hours, AAPH impaired the filterability so much that no-flow was observed under the filtration pressure of 70 mmH2O or less (Fig. 4), indicating that AAPH–damaged erythrocytes were stuck in the nickel mesh pores.

In the present study, MCV of erythrocytes exposed to 50 mM AAPH was increased in a time–dependent manner (Fig. 1), indicating that AAPH–induced oxidative stress caused gradual erythrocyte swelling. When the thickness of swollen erythrocytes increases, disc–like erythrocytes’ bending deformation inevitably becomes difficult, leading to time–dependent impairment of the filterability (Figs. 4 and 5). AAPH–induced oxidative stress increases erythrocyte membrane permeability for water and small ions such as K+ and Ca2+, which is supposed to be due to AAPH–induced progressive membrane phospholipid peroxidation and membrane leakage formation, because the time–dependent degradation of membrane phospholipid was confirmed in our previous study, that is 50 mM AAPH dramatically reduced α–tocopherol and γ–tocopherol to less than 50% and phosphatidylethanolamine to 80% of their initial levels. On the other hand, band 3 protein plays an important role in cellular volume regulation as an anion exchanger, and is considered to be another target of AAPH–induced oxidative stress.

The met–Hb formation as observed in this study (Fig. 2) indicates the interaction of AAPH liberating ROS with oxyhemoglobin and oxidation of the heme iron. This phenomenon is confirmed in the oxidant stress model of human erythrocytes using hypoxanthine–xanthine oxidase reaction generating superoxide anion. However, the application of superoxide to the resealed ghost of human erythrocytes did not cause any discernible membrane damage. This strongly indicates that intracellular met–Hb formation yields chain–reacting auto–oxidation of membrane components such as membrane protein degradation and membrane phospholipid peroxidation leading to membrane leakage and cellular swelling. Comparatively, percent met–Hb formation induced by lipophilic peroxidant tert–butylhydroperoxide (nearly 60%) is far greater than that induced by this hydrophilic AAPH (less than 10%) under different (0.5 mM vs. 50 mM) but equivalent doses with respect to the extent of hemolysis. This supports the assertion that the different membrane permeabilities and hence different locations of ROS generation between tert–butylhydroperoxide and AAPH underlie the different extents of met–Hb formation. Because hydrophobicity of a
drug influences its penetration of erythrocyte membrane or its location within the membrane phospholipid organization.

Erythrocyte swelling impairs the filterability (whole-cell bending deformability) by reducing the ratio of surface area to its volume. Oxidatively damaged erythrocytes cause K+ efflux which may allow concomitant water efflux. This may counteract the swelling of AAPH–treated erythrocytes. However, such membrane damage also allows Ca2+ and Na+ influx that balance the K+ efflux. Therefore, there seems to be no changes of the intracellular total cation content in the oxidatively damaged erythrocytes26). We speculate the membrane leak pathway other than ion–permeable channels, i.e., prelytic pores in the perturbed membrane may play a role in water influx and erythrocyte swelling. Scanning electron microscopic examination clarified gross morphological changes of membrane blebs, extrusions and ruffling in the AAPH–treated erythrocytes, suggesting nonspecific water–permeable leak pathway14).

An AAPH–evoked, time–dependent hemolysis was observed (Fig. 3) as in previous studies that applied the same concentration of AAPH (50 mM) to the human erythrocyte suspension with hematocrit ranging from 5 to 10% that is slightly higher than in this study (3%)13, 14). Oxidative hemolysis is a final event of extremely damaged erythrocytes, which was evident hemorheologically in this study (Fig. 4). As a matter of fact, AAPH–induced hemolysis is preceded by the markedly impaired filterability (Figs. 3 and 5). Human organisms possess several antioxidant defense mechanisms such as membrane α–tocopherol, internal enzymes such as catalase, superoxide dismutase and glutathione peroxidase. Lag of erythrocyte swelling (Fig. 1), met–Hb formation (Fig. 2), impairment of filterability (Fig. 5) and hemolysis (Fig. 3) after starting the application of AAPH may reflect the redox reaction of the defense system against extrinsic oxidant stress16).

This study has several limitations. First, hemolysis assay was performed in reaction mixture under static condition without any shear stress, and is not directly correlated to dynamic erythrocyte filterability. In that filtration process per se induces mechanical stress, the combined mechanical and oxidative stress may accelerate the erythrocyte damage which promotes hemolysis to a greater extent than that observed in the present study29. The second limitation is a lack of osmotic control in the reaction mixture of incubation with AAPH. However, AAPH–induced increase of MCV indicates that oxidative effects predominate over osmotic effects. The third limitation is that the reasons for the erythrocyte filterability impairment preceding the erythrocyte swelling remain unclear. More comprehensive protocol including erythrocyte oxidation such as malondialdehyde quantification is required to answer this question. Moreover, such mechanistic membrane study should include electron microscopic and fluorescent polarization techniques to investigate oxidatively damaged membrane structure and fluidity.

5. Conclusions

The present study indicates that oxidant stress induced by AAPH demonstrates time–dependent impairment of human erythrocyte deformability, erythrocyte swelling and met–Hb formation leading to oxidative hemolysis. This series of oxidative events are considered to be interdependent. Erythrocyte swelling causes geometric influences, and met–Hb induces chain–reacting oxidative membrane damage. These combined effects accelerate the impairment of AAPH–treated erythrocyte deformability. Our filtration technique clarified that hemorheological derangement precedes the oxidative hemolysis, which is relevant to in vivo microcirculation observed in vigorous athletes and subjects with unstable hemoglobin27, 28). However, prime factors initiating this hemorheological derangement remain unknown in this study.

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