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Method to Improve Azo-Compound (AAPH)-Induced Hemolysis of Erythrocytes for Assessing Antioxidant Activity of Lipophilic Compounds

Yusuke Nuruki,[†] Haruka Matsumoto,[‡] Miho Tsukada,[§] Haruka Tsukahara,[§] Tokuko Takajo, Kazunori Tsuchida, and Kazunori Anzai*

Division of Physical and Analytical Chemistry, Faculty of Pharmaceutical Sciences, Nihon Pharmaceutical University; Saitama 362–0806, Japan.

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We examined the method of oxidative hemolysis for assessment of antioxidant activity of various compounds, especially lipophilic compounds. 2,2'-Azobis(amidinopropane) dihydrochloride (AAPH) was used as the source of free radicals for the oxidative hemolysis of horse erythrocytes. We found that absorbance at 540 nm is not appropriate for monitoring AAPH-induced hemolysis. Instead, we should use absorbance at 523 nm (an isosbestic point), because AAPH oxidizes the oxygenated hemoglobin to methemoglobin and absorbance at 540 nm does not correctly reflect the amount of released hemoglobin by AAPH-induced hemolysis. The corrected method of AAPH-induced hemolysis was applicable to assess the antioxidant activity of various hydrophilic compounds such as ascorbic acid, (–)-epicatechin, and edaravone. For the assessment of antioxidant activity of lipophilic compounds, we need appropriate dispersing agents for these lipophilic compounds. Among several agents tested, 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposome at a concentration of 0.34 mM was found to be useful. Exogenous α -tocopherol incorporated using DMPC liposome as a dispersing agent was shown to protect erythrocytes from AAPH-induced hemolysis in a concentration-dependent manner.

Key words antioxidant; hemolysis; erythrocyte; 2,2'-azobis(amidinopropane) dihydrochloride (AAPH)

Introduction

Reactive oxygen species (ROS) attack various components of the living body, such as proteins, nucleic acids, and lipids, on which various harmful effects are exerted.¹⁾ To prevent the harmful effects of ROS, organisms have antioxidant enzymes in the body, such as superoxide dismutase (SOD) and peroxidase, as well as various antioxidative components such as α -tocopherol (α -Toc) and uric acid. In addition, various antioxidative compounds in foods, beverages, and nutritional supplements may be useful for preventing or reducing the harmful effects of ROS. A variety of techniques have been proposed to measure antioxidant activity of complex compound mixtures including ESR spin trapping technique,^{2,3)} oxygen radical absorbance capacity (ORAC) assay,^{4,5)} trolox equivalent antioxidant capacity (TEAC) assay,⁶⁾ and ferric reducing antioxidant power (FRAP) assay.⁷⁾

Lipid peroxidation of biomembranes is an initial step of oxidative cell damage. Therefore, it is possible to utilize lipid peroxidation as an indicator of oxidative stress. If we measure lipid peroxidation and related phenomena with and without a testing compound, we may evaluate the an-

tiioxidant activity of the testing compound. Previous studies reported that hemolysis of erythrocytes was induced by 2,2'-azobis(amidinopropane) dihydrochloride (AAPH), a water-soluble azo compound, when added to a suspension of erythrocytes and that this hemolysis was suppressed by antioxidants.^{8–11)} Oxidation of erythrocytes serves as a model for the oxidation damage of biological membranes and the free radicals generated in an aqueous phase induce chain oxidation of lipids and proteins in erythrocyte membranes that cause hemolysis.^{7,9,10)} AAPH is an oxidation agent, and thermal decomposition of an AAPH molecule generates two carbon-centered alkyl radical molecules, which then react with oxygen to produce peroxy radical molecules^{8,9,12)} and alkoxy radical molecules¹³⁾ with oxidation activity. Many studies have used AAPH-induced oxidative hemolysis to measure antioxidant activity of various compounds, especially complex mixture compounds.^{14–30)} In the present study, we re-examined the methodology of AAPH-induced oxidative hemolysis and propose an improved method for evaluating antioxidant activity of various compounds, especially lipophilic compounds, using AAPH-induced hemolysis of erythrocytes.

Results

When the erythrocyte suspension was mixed with the AAPH solution, absorbance at about 540 and 570 nm of the erythrocyte supernatant after centrifugation increased with time. The spectral pattern with absorption bands at these wavelengths corresponded to the spectrum of oxygenated hemoglobin (HbO₂),^{31–33)} indicating that hemolysis was induced by the reaction with AAPH. In addition to the increase in the

[†]Present address: Kawaguchi Kogyo General Hospital; 1–18–15 Aoki, Kawaguchi, Saitama 332–0031, Japan.

[‡]Present address: Matsumotokiyo Co. Ltd.; 9–1 Shinmatsudohigashi, Matsudo, Chiba 270–8501, Japan.

[§]Present address: Shichihongi Pharmacy; 1904–1 Shichihongi, Kamisato-machi, Kodama-gun, Saitama 369–0306, Japan.

[§]Present address: Chuoh Pharmacy; 11–21 Minamitasago-cho, Utsunomiya, Tochigi 321–0143, Japan.

*To whom correspondence should be addressed. e-mail: anzai@nichiyaku.ac.jp

Fig. 1. Effects of AAPH on Erythrocytes

A) Hemoglobin was released from horse blood by hemolysis of erythrocytes with water, and then AAPH was added. Time course absorption spectra were measured at 0, 15, 30, 45, 60, 75, and 90 min after mixing with AAPH. The absorbance around 550 nm decreased with time, whereas absorbance around 630 nm increased. Two isosbestic points shown in circles were observed at 523 and 591 nm. B) A conceptual diagram of the effects of AAPH on erythrocytes. An AAPH molecule is thermally decomposed to two molecules of 2-methyl-amidinopropane radicals, which are converted to peroxy radicals ($\text{ROO}^\bullet$) by the reaction with a molecular oxygen and alkoxyl radicals ($\text{RO}^\bullet$) by the decomposition of $\text{ROO}^\bullet$. $\text{ROO}^\bullet$ and $\text{RO}^\bullet$ induce hemolysis by oxidative membrane damage of erythrocytes as the first step. $\text{ROO}^\bullet$ and $\text{RO}^\bullet$ also oxidize the released hemoglobin (HbO_2) with Fe^{2+} in the heme group to methemoglobin (metHb) with Fe^{3+} heme as the second step.

two peaks, we found that a new absorption band appeared at 630 nm after 90 min of the reaction. When the spectrum of the sample obtained from the complete hemolysis of erythrocytes induced by the addition of water in the presence of AAPH was compared with that in the absence of AAPH, we found that the intensity of the two peaks of the AAPH-treated erythrocytes were lower than that of the untreated erythrocytes. This observation suggested that a new species was produced by the reaction of HbO_2 with AAPH. To examine this further, we first prepared a sample of complete hemolysis by treating erythrocytes with water, and then AAPH was added. As shown in Fig. 1A, the intensity of the bands at 541 nm and 576 nm decreased with time, while the intensity of the newly observed band at 630 nm increased. The reaction of AAPH was not very fast. Indeed, a small change was observed in the absorbance at 541 and 576 nm even at 1 d after mixing, indicating that the reaction did not reach equilibrium (data not shown). Two isosbestic points were observed at 523 and 591 nm. If we measure the absorbance at 541 or 576 nm, the

Fig. 2. Hemolysis of Erythrocytes by Treatment with AAPH and Inhibition by Ascorbic Acid

Open circles (○) represent the hemolysis of erythrocytes caused by AAPH-induced oxidative membrane damage. The final reaction mixture comprised of 10% erythrocytes and 50 mM AAPH. Hemolysis was suppressed by ascorbic acid (●: 80 μM , ▲: 240 μM). The reaction temperature was 37 °C. The extent of hemolysis (%) was obtained using the ratio of the absorbance at 523 nm of the supernatant aliquot sample to that of the completely hemolyzed sample (water-treated). A typical result in multiple experiments is shown.

change in the absorbance with time is informative about both hemolysis and reaction of released HbO_2 with AAPH (Fig. 1B). Instead, measuring the absorbance at 523 or 591 nm gives us information that only reflects the hemolysis induced by AAPH. Using the absorbance of the isosbestic point at 523 nm is preferable to 591 nm because intensity at 523 nm is larger than that at 591 nm.

A typical example of the time course of hemolysis induced by AAPH is shown in Fig. 2. Hemolysis began to increase at around 60 min, greatly increased at around 75–90 min, and reached a plateau at around 120 min. Addition of ascorbic acid delayed the beginning of hemolysis dose-dependently. Some other hydrophilic antioxidants, such as uric acid and α -lipoic acid, also induced a similar delay in hemolysis, although the effective concentration range differed amongst each other (data not shown).

(–)-Epicatechin and edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut®), which are also hydrophilic antioxidants, showed stronger suppression against AAPH-induced hemolysis than ascorbic acid did (Fig. 3). For the sample of 80 μM (–)-epicatechin, the absorbance at 523 nm began to increase at 165 min due to hemolysis, whereas no increase was observed for the sample of 80 μM edaravone. The suppression effect against AAPH-induced hemolysis was in the order edaravone > (–)-epicatechin > ascorbic acid. (–)-Epicatechin showed the absorbance at 523 nm at early time, but the absorbance decreased after the maximum (at 30 min for 80 μM and 75 min for 240 μM). These absorption at 523 nm observed at early time is not due to hemolysis because it decreased in time and the absorption spectrum was different from that observed by the hemolysis with AAPH (data not shown).

In order to assess the antioxidant activity of lipophilic compounds using the method of hemolysis of erythrocytes, we searched for appropriate agents that disperse lipophilic compounds to incorporate into the erythrocyte membranes. Dimethyl sulfoxide (DMSO), methylcellulose, and Tween 20 were examined, but all were found to be unsuitable. DMSO has antioxidant activity by itself, and methylcellulose and

Fig. 3. Effects of (–)-Epicatechin and Edaravone on the Time Course of AAPH-Induced Hemolysis

Hemolysis was induced by 50 mM AAPH at 37°C. A) Open circles (○) represent the hemolysis caused by AAPH-induced oxidative membrane damage in the absence of (–)-epicatechin. Closed circles (●) represent the hemolysis induced by AAPH in the presence of 80 μM (–)-epicatechin. Closed triangles (▲) represent the hemolysis induced by AAPH in the presence of 240 μM (–)-epicatechin. B) Open circles (○) represent the hemolysis caused by AAPH-induced oxidative membrane damage in the absence of edaravone. Closed circles (●) represent the hemolysis induced by AAPH in the presence of 80 μM edaravone. Closed triangles (▲) represent the hemolysis induced by AAPH in the presence of 240 μM edaravone. The extent of hemolysis (%) was obtained using the ratio of the absorbance at 523 nm of the supernatant aliquot sample to that of the completely hemolyzed sample (water-treated). A typical result in multiple experiments is shown.

Tween 20 have strong detergent activity that induced hemolysis even before treatment with AAPH (data not shown). Next, we tried liposomes prepared with soybean phospholipids (asolec-tin) or 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine (DMPC). Asolec-tin liposomes (as low as 0.75 mM in concentration) accelerated the hemolysis induced by AAPH (data not shown). In contrast, DMPC liposomes (up to 0.74 mM) did not accelerate the AAPH-induced hemolysis. Figure 4 shows the effect of 0.34 mM DMPC liposomes on the time course of hemolysis induced by 50 mM AAPH.

Based on the above observations, we examined the anti-oxidant effect of a typical lipophilic antioxidant, α -tocopherol (α -Toc), on hemolysis using DMPC liposomes as a dispersing agent. DMPC liposomes at 0.34 mM were used as a dispersing agent for various concentrations of α -Toc in an 8.3% erythrocyte suspension. As shown in Fig. 5, the hemolysis induced by AAPH was inhibited by pretreatment of erythrocytes with

Fig. 4. Effect of DMPC Liposomes on the Time Course of AAPH-Induced Hemolysis

Hemolysis was induced by 50 mM AAPH at 37°C for the erythrocyte suspension with or without DMPC liposomes. Closed circles (●) represent the time-course of the AAPH-induced hemolysis of erythrocytes without DMPC liposome treatment, and open circles (○) represent the time-course of the AAPH-induced hemolysis of erythrocytes treated with 0.34 mM DMPC liposomes. The extent of hemolysis (%) was obtained using the ratio of the absorbance at 523 nm of the supernatant aliquot sample to that of the completely hemolyzed sample (water-treated). Each point represents an average of three experiments and the bars represent standard deviation.

Fig. 5. Effect of α -Toc on the Time Course of AAPH-induced Hemolysis

Hemolysis was induced by 50 mM AAPH at 37°C. The erythrocyte suspension was treated with different concentrations of α -Toc dispersed in 0.34 mM DMPC liposomes. Closed circles (●) represent the time course of erythrocyte hemolysis without treatment with α -Toc containing DMPC liposomes. The erythrocyte suspension was treated with DMPC liposomes containing 18 μM α -Toc (○), 46 μM α -Toc (Δ), and 182 μM α -Toc (*). The extent of hemolysis (%) was obtained using the ratio of the absorbance at 523 nm of the supernatant aliquot sample to that of the completely hemolyzed sample (water-treated). A typical result in multiple experiments is shown.

α -Toc dispersed in DMPC liposomes. This effect was dependent on the concentration of α -Toc.

Discussion

Hemolysis of erythrocytes is often measured by the optical absorbance of released HbO₂. The wavelengths used for measuring the extent of hemolysis are around 400 nm (Soret-band) and 500–600 nm (Q-band). The absorption band at about 540 nm has often been used,³⁴ and use of this wavelength is reasonable because the band is relatively broad and displays sufficient intensity.

Thermal decomposition of AAPH firstly produces peroxy

radical ($\text{ROO}^\bullet$) and further reaction produces alkoxyl radical ($\text{RO}^\bullet$), which is suggested as the major species for the oxidation reaction of substrates.¹³⁾ For AAPH-induced oxidative hemolysis experiments, most of the published studies to date also used absorbance at about 540 nm to analyze the extent of hemolysis.^{8,10,11,14–30)} The absorption spectrum of erythrocytes that we observed after water-induced hemolysis had peaks at 541 and 576 nm (Fig. 1A). These two absorption maxima are derived from oxygenated hemoglobin (HbO_2), as reported previously.^{31–33)} As shown in Fig. 1B, HbO_2 with a Fe^{2+} heme in the hemoglobin should be oxidized by AAPH, producing methemoglobin (metHb) with Fe^{3+} heme (an oxidized hemoglobin) after release from erythrocytes by hemolysis.³⁵⁾ An absorption band at 630 nm was reported for metHb.^{31–33)} Since the absorbance at 540 nm of metHb is lower than that of HbO_2 (Fig. 1A) and the formation of metHb is not very fast, we should not use the wavelength of about 540 nm to monitor the extent of hemolysis caused by AAPH. As shown in Fig. 1A, clear isosbestic points were observed at 523 and 591 nm, indicating that absorbance at these wavelengths correctly reflects the amount of released hemoglobin regardless of the status of hemoglobin (oxidized or not oxidized). We used absorbance at 523 nm for the analysis of hemolysis in this study because the intensity at 523 nm is larger than that at 591 nm.

We confirmed that AAPH induces oxidative hemolysis, as previously reported.^{8,10,11)} The hemolysis induced by AAPH was suppressed by ascorbic acid and uric acid dose-dependently, as reported by Niki *et al.*⁸⁾ It is reasonable to explain the suppression as follows: peroxy radicals generated from AAPH were scavenged by ascorbic acid or uric acid in the aqueous phase until the antioxidant was used up. (–)-Epicatechin also showed dose-dependent antioxidant activity, as reported by Grzesik *et al.*³⁶⁾ The activity was stronger than ascorbic acid. Edaravone also showed suppression of hemolysis induced by AAPH; this compound showed a very strong effect even at $80\text{ }\mu\text{M}$ probably because it is a good scavenger of peroxy radicals.³⁷⁾

There are many methods for measuring antioxidant activities of water-soluble compounds. However, only a relatively small number of methods have been reported to monitor antioxidant activity of lipophilic compounds.^{38–40)} Therefore, we attempted to identify a condition for applying the method of AAPH-induced oxidative hemolysis for assessing antioxidant activity of lipophilic compounds. Initially, several agents were examined to find appropriate agents for dispersing lipophilic compounds. Among them, DMPC liposome was found to be suitable for dispersing lipophilic compounds and incorporating them into erythrocytes membranes. DMPC liposome has been used previously by Niki *et al.*,⁸⁾ but they observed enhancement of AAPH-induced hemolysis by 1.84 mM DMPC liposome. In the present study, we also observed enhancement of AAPH-induced hemolysis by DMPC liposomes at similar concentrations, but lowering the concentration of DMPC liposomes improved the results; *i.e.*, there was no effect on hemolysis. We found that concentrations lower than 0.74 mM did not affect AAPH-induced hemolysis (Fig. 4).

This result was adopted to assess the method of oxidative hemolysis for testing the antioxidant effect of lipophilic compounds, using α -Toc as a typical lipophilic antioxidant. AAPH-induced hemolysis was suppressed by the α -Toc that was incorporated into the erythrocyte membranes by DMPC

liposomes. α -Toc as low as $18\text{ }\mu\text{M}$ was effective for suppressing the AAPH-induced hemolysis. If we assume that all 0.34 mM DMPC molecules and $18\text{ }\mu\text{M}$ α -Toc molecules were incorporated evenly in the erythrocyte membranes of 8.3% erythrocyte suspension, then one erythrocyte is calculated to have about 5×10^6 DMPC molecules and about 3×10^4 α -Toc molecules. The inhibitory effect of α -Toc against AAPH-induced hemolysis was reported by Miki *et al.* by comparing normal erythrocytes and α -Toc deficient erythrocytes.¹¹⁾ In the present study, we also found that exogenously-applied α -Toc, by the use of DMPC liposomes, suppressed the AAPH-induced oxidative hemolysis.

In conclusion, we showed in the present study that the method of AAPH-induced oxidative hemolysis for assessing antioxidant and membrane-protection activities of lipophilic compounds may be improved by using: (1) the absorbance at 523 nm for monitoring AAPH-induced hemolysis, and (2) 0.34 mM DMPC liposomes for dispersing the lipophilic compounds to apply to erythrocyte suspensions.

Experimental

Chemicals DMPC, asolectin, and α -Toc were obtained from Sigma-Aldrich Co., Ltd., U.S.A. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut[®]) was a gift from Mitsubishi Pharma Co. (Tokyo, Japan). All other reagents were obtained from Wako Pure Chemical Corporation, Japan. All reagents were of the highest grade available and used without further purification.

Preparation of 20% Erythrocyte Suspension Fresh horse blood was obtained from Nippon Bio-test Laboratories Inc. (Saitama, Japan) and diluted to a 20% suspension with 10 mM phosphate buffered saline (pH 7.4) (PBS). The suspension was centrifuged at $2000 \times g$ for 5 min at room temperature, and erythrocytes were separated from the plasma and buffy coat. The pellet was resuspended with PBS to the original volume. This washing process was repeated three times and 20% erythrocyte suspension was prepared.

Measurement of Effect of Hydrophilic Antioxidants on AAPH-Induced Hemolysis of Erythrocytes The 20% erythrocyte suspension with or without hydrophilic antioxidants and a solution of 100 mM AAPH in 10 mM PBS were pre-incubated separately at 37°C for 5 min in a water bath. The erythrocyte suspension (5 mL) was mixed with the same volume of AAPH solution and incubated in a water bath at 37°C in room light. The final concentrations were 10% erythrocytes and 50 mM AAPH. An aliquot (0.70 mL) was taken out periodically and centrifuged at $5000 \times g$ for 2 min. Certain volumes of the supernatant were taken out and diluted with water to examine the extent of hemolysis by measuring the absorbance at 523 nm with a UV-visible spectrophotometer (Shimadzu UV-2550, Japan). Complete hemolysis was performed by diluting the erythrocyte suspension 10-fold with water. The extent of hemolysis (%) was calculated as the ratio of the absorbance at 523 nm of the sample to that of the complete hemolysis sample. For the experiments investigating the effect of hydrophilic antioxidants, the antioxidants were firstly mixed with the erythrocyte and then the AAPH solution was added.

Measurement of Effect of α -Toc on AAPH-Induced Hemolysis of Erythrocytes For the experiments investigating the effect of hydrophobic α -Toc, several reagents for suspend-

ing α -Toc were initially trialed and DMPC was found to be appropriate. Therefore, α -Toc was suspended in DMPC liposomes, which were then applied to the erythrocyte suspension before mixing with the AAPH solution. Usually, a certain concentration of α -Toc in chloroform was mixed with a chloroform solution of DMPC in a flask and then the chloroform was evaporated using a rotary evaporator. PBS was added to the flask and the mixture was sonicated for several minutes in a nitrogen atmosphere to make DMPC liposomes containing α -Toc. The erythrocyte suspension (20%, 2.5 mL) was mixed with 0.5 mL of DMPC liposomes containing certain amounts of α -Toc, or without α -Toc, and incubated for 15 min at 37°C, and then centrifuged at $2000 \times g$ for 5 min. The supernatant was removed and the pellet was resuspended in 10 mM PBS up to the original volume (3 mL). The suspension (3 mL) was mixed with the same volume of 100 mM AAPH solution and incubated in a water bath at 37°C. The final concentrations were 8.3% erythrocytes, 50 mM AAPH, and DMPC. An aliquot (0.50 mL) was taken out periodically to measure the extent of hemolysis with the UV-visible spectrophotometer (Shimadzu UV-2550, Japan).

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Conflict of Interest The authors declare no conflict of interest.

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