FEBS Letters 413 (1997) 236–238 FEBS 19070

Peroxynitrite-induced hemolysis of human erythrocytes and its inhibition by antioxidants

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Received 24 June 1997; revised version received 4 July 1997

Abstract It was found that human erythrocytes underwent hemolysis when incubated with peroxynitrite at 37°C under air. The extent of hemolysis increased with increasing peroxynitrite concentration and decreasing hematocrit. The peroxynitrite-induced hemolysis was suppressed only partially by a radical scavenging antioxidant such as uric acid and Trolox, a water-soluble vitamin E analogue, but reduced glutathione, *N*-acetylcysteine and albumin efficiently inhibited the hemolysis. A selenium-containing organic compound, ebselen, also suppressed the hemolysis. On the other hand, nitric oxide and superoxide generated concomitantly from 3-morpholinosydnonimine (SIN-1) did not induce appreciable hemolysis, while it converted hemoglobin to methemoglobin extensively.

Key words: Nitric oxide; Peroxynitrite; Hemolysis; Antioxidants

1. Introduction

Nitric oxide (NO) is, like oxygen, a double-edged sword. It exerts diverse biological functions such as blood pressure modulation, vasodilation, neurotransmission and inhibition of platelet adherence and aggregation, but it also mediates tissue injury and may contribute to the pathology of neurodegenerative disease, inflammation, and atherosclerosis [1,2]. NO is not reactive per se, but it reacts with superoxide rapidly to give peroxynitrite, $ONOO^-$, which induces various oxidative damage [3]. NO together with superoxide induces lipid peroxidation [4,5], but NO also acts as a potent peroxyl radical scavenger and inhibits lipid peroxidation [6–10].

The oxidation of erythrocytes has been studied extensively as a model of oxidative damage of biomembranes [11]. For example, it has been shown that free radicals attack erythrocyte membranes to induce the oxidations of lipids and proteins and eventually cause hemolysis and that such hemolysis is suppressed by vitamin E and vitamin C [12–15]. In the present study, it was found that peroxynitrite was capable of inducing hemolysis of human erythrocytes which was suppressed efficiently by albumin and glutathione.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; NAC, *N*-acetylcysteine; NO, nitric oxide; PBS, phosphate buffered saline; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; SIN-1, 3-morpholinosydnonimine

2. Materials and methods

2.1. Materials

Fresh, heparinized blood from a healthy donor was centrifuged at $1000 \times g$ for 10 min and erythrocytes were separated from plasma and buffy coat and washed three times with a physiological saline. Peroxynitrite was synthesized from hydrogen peroxide and KNO_2 in phosphate buffered saline (pH 7.4) as described in the literature [2]. After quenching of the reaction with ice cold NaOH, the top layer of the solution was collected and the concentration of peroxynitrite was determined from an absorption at 302 nm and a molar extinction of 1670 M^{-1} cm^{-1} [2]. The remaining hydrogen peroxide was removed with MnO_2. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Pure Chemical Ind. (Osaka, Japan). Ebselen, 2-phenyl-1,2-bezisoselenazol-3(2H)-one, was a kind gift from Dr. Masayasu at Daiichi Pharmaceutical Co. (Tokyo, Japan). Other chemicals were of the highest grade available commercially.

2.2. Methods

The erythrocyte suspensions in physiological saline containing 30 mM phosphate buffer (pH 7.4) were incubated at 37°C in air and an aliquot was taken out periodically to measure the extent of hemolysis spectrophotometrically as described in the previous paper [12]. The consumption of endogenous α-tocopherol was measured with an HPLC equipped with an electrochemical detector using LC-18 column (Supelco) and methanol/tert-butyl alcohol (90:10 v/v) containing 50 mM $NaClO_4$ as an eluent at a rate of 1.0 m/min [16]. The electrochemical detector (Shiseido Nanospace SI-1, Tokyo) was set at +800 mV. The amount of reduced glutathione was measured with Ellman's reagent as described in the literature [17]. Phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) were measured by a chemiluminescence-based high performance liquid chromatography assay [18].

3. Results

It was found that human erythrocytes underwent hemolysis when incubated with peroxynitrite at 37°C under air in physiological saline (pH 7.4). The hemolysis was observed rapidly after addition of peroxynitrite and then increased only gradually with time (data not shown). Little hemolysis was observed in the absence of peroxynitrite. Furthermore, it was confirmed that the addition of peroxynitrite solution after standing at room temperature and pH 7.4 for 1 h did not induce hemolysis, suggesting that the species such as nitrite, nitrate and hydrogen peroxide which might contaminate the solution are not responsible for hemolysis. The endogenous α-tocopherol decreased rapidly immediately after the addition of peroxynitrite but then remained unchanged. A similar phenomenon was observed with reduced glutathione (data not shown). The extent of hemolysis increased with increasing concentration of peroxynitrite (Fig. 1).

The endogenous α-tocopherol and glutathione in the erythrocytes were consumed during incubation with peroxynitrite in a concentration-dependent manner (Fig. 2). The amounts of α-tocopherol and glutathione consumed were smaller than

PII S0014-5793(97)00922-8

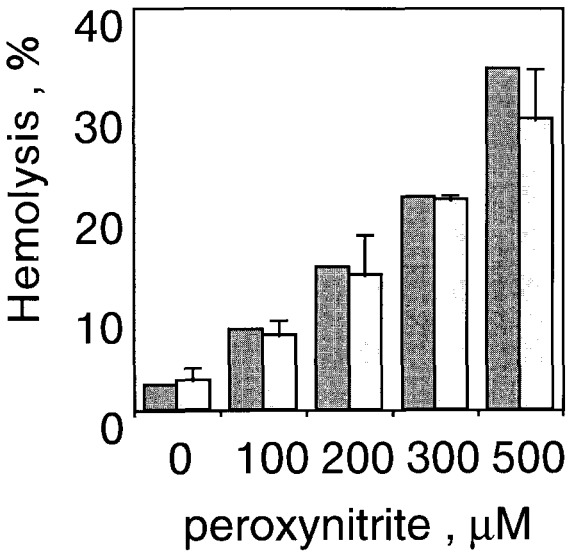

Fig. 1. Effects of peroxynitrite concentration and hematocrit on the hemolysis. Human erythrocytes were incubated with different concentrations of peroxynitrite at 37°C in air for 30 min and the extent of hemolysis was measured as described in Section 2.2. Hematocrit: dark-saded boxes, 5%; light-shaded boxes, 10%. Values are means ± S.E. of 3 to 4 independent experiments.

the initial amount of peroxynitrite added and the erythrocytes underwent hemolysis while a considerable amount of α-tocopherol still remained.

The effects of various antioxidants against hemolysis of human erythrocytes induced by peroxynitrite were studied and the results are summarized in Fig. 3. Trolox, a water-soluble analogue of vitamin E which acts as a potent radical scavenger, uric acid and catalase were not very effective but glutathione, *N*-acetylcysteine and albumin suppressed the hemolysis completely. Ebselen, a synthetic selenillm-containing compound, was also effective in suppressing the hemolysis, although it increased hemolysis by itself.

It has been found previously that AAPH which generates aqueous peroxyl radicals by thermal decomposition in air induces hemolysis [12–15]. In the present study the hemolysis induced by peroxynitrite and AAPH was compared at the point when about the same extent of hemolysis took place. The results are summarized in Table 1. Peroxynitrite (300 μM) induced 21% hemolysis in 1 h, while AAPH (150 mM) induced 26% hemolysis in 2.5 h. Since the half-life of peroxynitrite under the present conditions is about 1 s [2], it is assumed that all of peroxynitrite is decomposed in 1 h. The amount of free radicals (aqueous peroxyl radicals) formed from AAPH is calculated from $1.30\times10^{-6}\times$AAPH (M)$\times$time (s) [19] and it is obtained as $1.30\times10^{-6}\times0.150\times2.5\times3600=1755$ μM in 2.5 h. Substantially all of the endogenous α-tocopherol and glutathione were consumed in the AAPH-induced oxidation, but considerable amounts were still remaining in the oxidation induced by peroxynitrite. The most notable difference between the reactions induced by peroxynitrite and AAPH is the formation of lipid hydroperoxide. Phosphatidylcholine hydroperoxide was not detected in the peroxynitrite-induced oxidation, while a considerable amount was found in the oxidation induced by AAPH. Little phosphatidylethanolamine hydroperoxide was formed in both oxidations.

3-Morpholinosydnonimine (SIN-1) is known to generate spontaneously both superoxide and nitric oxide concomitantly under physiological conditions and can be used as a convenient source of peroxynitrite [20]. It was observed that phosphatidylcholine hydroperoxide was formed when liposomal membranes were incubated with SIN-1 (data not shown), but little hemolysis took place when human erythrocytes were incubated with SIN-1 at concentrations as high as 10 mM. Instead, the formation of methemoglobin was observed, apparently by reacting with nitric oxide. On the other hand, the extent of methemoglobin formation by peroxynitrite was small.

4. Discussion

It was found previously that the aqueous peroxyl radicals derived from AAPH attack erythrocyte membranes to induce the oxidations of phospholipids, protein and α-tocopherol, which eventually lead to hemolysis [12–15]. The extent of hemolysis was directly proportional to the amount of peroxyl radicals formed [14]. As shown above, peroxynitrite induced hemolysis of human erythrocytes in a concentration-dependent manner. The hemolysis induced by peroxynitrite and AAPH has similarities and dissimilarities. One of the major differences between the hemolysis induced by two kinds of different oxidants is the formation of phosphatidylcholine hydroperoxide. No hydroperoxide was observed in the peroxynitrite induced oxidation, whereas a considerable amount was observed in the oxidation induced by AAPH.

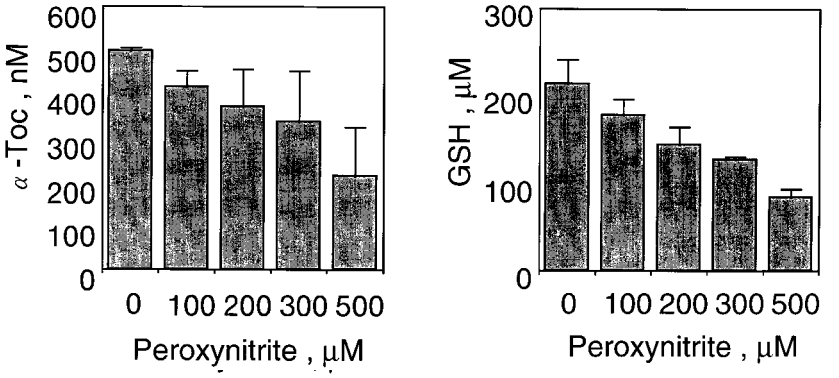

Fig. 2. Consumption of α-tocopherol and reduced glutathione during hemolysis induced by peroxynitrite. Human erythrocytes (10% hematocrit) were incubated with peroxynitrite at 37°C in air for 60 min and the concentrations of remaining α-tocopherol and glutathione were measured as described in Section 2.2. Values are means ± S.E. of 3 to 4 independent experiments.

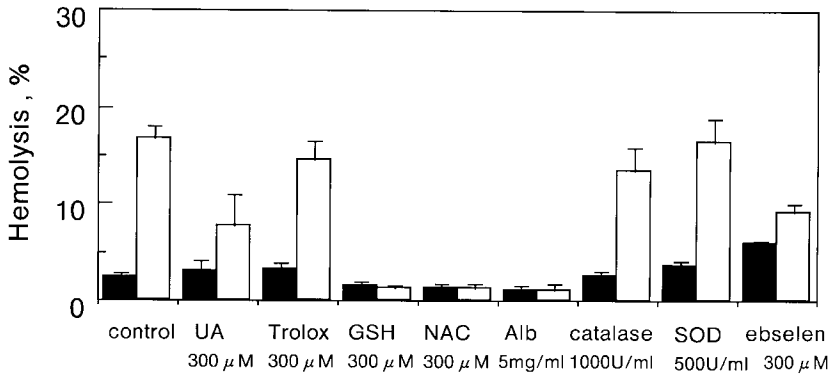

Fig. 3. Effects of antioxidants against hemolysis of human erythrocytes induced by peroxynitrite. Human erythrocytes (10% hematocrit) were incubated with 100 μM peroxynitrite in the absence and presence of antioxidant in physiological saline (pH 7.4) at 37°C in air for 60 min and the extent of hemolysis was measured as described in Section 2.2. Black boxes, extent of hemolysis without peroxynitrite but with antioxidant. Open boxes, extent of hemolysis with peroxynitrite and antioxidant. Control, without antioxidant. Mean ± S.D., $n = 3$; UA, uric acid; GSH, glutathione; NAC, *N*-acetylcysteine; Alb, albumin; SOD, superoxide dismutase.

The facts that phosphatidylcholine hydroperoxide (PCOOH) was formed but that little phosphatidylethanolamine hydroperoxide (PEOOH) was formed in the AAPH-induced oxidation of erythrocytes suggest that the radicals attack erythrocyte membranes from outside. On the other hand, it has been reported that more PEOOH was formed than PCOOH during the oxidative damage of erythrocytes induced by superoxide [21]. In the present study, it was found that peroxynitrite induced hemolysis without appreciable lipid peroxidation. Probably peroxynitrite and/or the active species derived from it attack proteins more preferentially than lipids in the erythrocyte membranes. Thus, the mode of oxidative damage of erythrocyte membranes depends on the oxidants.

The efficiency of antioxidant also depends on the oxidants. The water-soluble radical-scavenging antioxidants such as ascorbic acid and Trolox are effective against AAPH-induced oxidations, whereas glutathione, which is less reactive toward peroxyl radicals than Trolox, does not act as an efficient inhibitor [13]. On the other hand, glutathione exerted much more potent activity than Trolox and uric acid (Fig. 3) in suppressing the hemolysis induced by peroxynitrite. Whiteman and Halliwell [22] observed that glutathione was effective in protecting against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation and Salgo and Pryor reported that Trolox inhibited peroxynitrite-induced apoptosis in rat thymocytes [23]. The formation of urate radical has been reported in the reaction with peroxynitrite [22].

Ebselen is a selenium-containing compound which exerts glutathione peroxidase activity [24], thereby acting as a potent antioxidant against metal-induced oxidation. It was observed in the present study that ebselen suppressed the peroxynitrite-induced hemolysis. This may be accounted for by the recent finding by Matsumoto and Sies [25] that ebselen acts as a potent scavenger of peroxynitrite.

In conclusion, the present study shows that, although the details of the underlying mechanism are not clear, peroxynitrite is capable of inducing hemolysis of erythrocytes. However, it may be noteworthy that SIN-1 which generates both superoxide and nitric oxide concomitantly did not induce hemolysis, implying that hemoglobin might act as a scavenger of nitric oxide [26] to inhibit the formation of peroxynitrite and accordingly hemolysis as well.

References

[1] Moncada, S. and Higgs, E.A. (1993) New Engl. J. Med. 329, 2002–2011.
[2] Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) Methods Enzymol. 233, 229–240.
[3] Pryor, W.A. and Squadrito, G.L. (1995) Am. J. Physiol. 268, L699–L722.
[4] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) Arch. Biochem. Biophys. 288, 481–487.
[5] Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T. and Moncada, S. (1992) Free Rad. Res. Commun. 17, 9–20.
[6] Rubbo, H., Radi, R., Truyjillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M. and Freeman, B.A. (1994) J. Biol. Chem. 269, 26066–26075.
[7] Jessup, W., Mohr, D., Gieseg, S.P., Dean, R.T. and Stocker, R. (1992) Biochim. Biophys. Acta 1180, 73–82.
[8] Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A. and Parthasarathy, S. (1993) FEBS Lett. 334, 170–174.
[9] Malo-Rante, U., Yla-Herttuala, S., Metsa-Ketela, T., Jaakkola, O., Moilanen, E., Vourimen, P. and Nikkari, T. (1994) FEBS Lett. 179–183.
[10] Hayashi, K., Noguchi, N. and Niki, E. (1995) FEBS Lett. 370, 37–40.
[11] Chiu, D., Lubin, B. and Shohet, S.B. (1982) in: Free Radicals in Biology, Vol. V (Pryor, W.A., Ed.), pp. 115–160.
[12] Yamamoto, Y., Niki, E., Kamiya, Y., Miki, M., Tamai, M. and Mino, M. (1986) J. Nutr. Sci. Vitaminol. 32, 475–479.
[13] Niki, E., Komuro, E., Takahashi, M., Urano, S., Ito, E. and Terao, K. (1988) J. Biol. Chem. 263, 19809–19814.
[14] Miki, M., Tamai, H., Mino, M., Yamamoto, Y. and Niki, E. (1987) Arch. Biochem. Biophys. 258, 373–380.
[15] Sandhu, I.S., Ware, K. and Grisham, M.B. (1992) Free Rad. Res. Commun. 16, 111–122.
[16] Miki, M., Tamai, H., Mino, M., Yamamoto, M. and Niki, E. (1987) Arch. Biochem. Biophys. 258, 373–380.
[17] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[18] Yamamoto, Y. (1994) Methods Enzymol. 233, 319–332.
[19] Niki, E. (1990) Methods Enzymol. 186, 100–108.
[20] Feelish, M., Ostrocoski, J. and Noak, E.A. (1989) J. Cardiovasc. Pharmacol. 14, S13–S22.
[21] Mino, M., Miki, M., Tamai, H., Yasuda, H. and Maeda, H. (1988) in: Lipid Peroxidation in Biological Systems (Sevanian, A., Ed.), pp. 51–70, American Oil Chemists' Society, Champain, Illinois.
[22] Whiteman, M. and Halliwell, B. (1996) Free Rad. Res. 25, 275–283.
[23] Salgo, M.G. and Pryor, W.A. (1996) Arch. Biochem. Biophys. 333, 482–488.
[24] Sies, H. (1993) Free Rad. Biol. Med. 14, 313–323.
[25] Masumoto, H. and Sies, H. (1996) Chem. Res. Toxicol. 9, 262–267.
[26] Jia, L., Bonaventura, C., Bonaventura, J. and Stamler, J.S. (1996) Nature 380, 221–226.