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GENERATION OF ACTIVE OXYGEN SPECIES IN BLOOD PLATELETS — SPIN TRAPPING ANALYSIS

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Generation of active oxygen species by bovine blood platelets was examined by the electron spin resonance (ESR) spin trapping technique with 5,5-dimethyl-1-pyroline-1-oxide (DMPO). The hydroxyl spin-trapped adduct 5,5-dimethyl-2-hydroxy-1-pyrolidinyloxy (DMPO-OH) was formed in the presence of platelets, indicating the generation of hydroxyl radicals (· OH) by the platelets. Generation of · OH was observed even with platelets in the resting state, but was markedly enhanced when the platelets were activated with stimulants. Stronger stimulants such as the calcium ionophore ionomycin, induced greater radical generation than the weaker stimulant ADP. When the platelets were stimulated by thrombin, generation of · OH was greatest after 1.5 min, and depended on the dose of the stimulant. It was inhibited by inhibitors of platelet activation such as forskolin and phenolic antioxidants.

KEY WORDS: blood platelets, active oxygen species, hydroxyl radical, spin trapping, DMPO(5,5-dimethyl-1-pyroline-1-oxide), platelet activation.

ABBREVIATIONS: DMPO, 5,5-dimethyl-1-pyroline-1-oxide; DMPO-OH, 5,5-dimethyl-2-hydroxy-1-pyrolidinyloxy; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2.

INTRODUCTION

The generations of active oxygen species are involved in various biological reactions and various kinds of diseases.[1-3] Neutrophils are known to generate superoxide anion radicals (O_2^-) as the result of activation of an NADPH-oxido-reductase[4] and these radicals play an important role in the function of these cells. Blood platelets have also been shown to generate active oxygen species when activated by various stimulants and these active oxygens have been suggested to be involved in injury of arteries and asthma.[5-7] However, little is known about the characteristics of generation of active oxygen species by blood platelets. Moreover, there have been no studies on the generation of active oxygen species by the ESR spin-trapping technique, by which a variety of free radical species can be distinguished.[8] Therefore, in this work we examined the generation of active oxygen species in blood platelets by the ESR technique with 5,5-dimethyl-1-pyroline-1-oxide (DMPO) as a spin trapping agent.

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MATERIALS AND METHODS

Platelet-rich plasma of bovine (Holstein) blood was obtained as described previously.[9] The plasma was centrifuged at 1,000 × g for 10 min, and the platelets separated were suspended in a solution of sodium/potassium-Tris medium (137 mM NaCl/5.4 mM KCl/11 mM dextrose/25 mM Tris-HCl adjusted to pH 7.4). Spontaneous platelet aggregation during preservation was prevented by adding 129 mM citrate (adjusted to pH 7.4) to this suspension at a volume ratio of 1 : 9.

After addition of diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid at 0.1 mM, a stimulant was added. DMPO was added to this suspension at a final concentration of 0.45 M. The final platelet concentration was 4.4 × 10^6 cells/μL. ESR spectra were measured with a JEOL JES-FE1XG (X-band) spectrometer with 100 kHz field modulation frequency and 1 G modulation amplitude at an out-put power of 8 mW. Mn(II) in MnO was used as a standard. All experiments were done at room temperature (21°C).

Platelet aggregation induced by thrombin in the presence of 0.2 mM $CaCl_2$ was examined in a RAM-11 aggregometer (Rikadenki Kogyo, Tokyo, Japan). Initial aggregation rates were measured as reported previously.[10]

RESULTS

First we measured the ESR spectra of resting and activated platelets. Resting platelets gave signals of low intensity, as shown in Figure 1(a). After addition of a stimulant such as ADP, thrombin or ionomycin, the signal intensities increased markedly (Figure 1(b)–(d)). The intensity ratio of the signals was 1 : 2 : 2 : 1, which is typical for the hydroxyl spin-trapped adduct, DMPO-OH, indicating the generation of hydroxyl radicals (· OH) by the platelets. These results provide first ESR evidence on the generation, which are consistent with the speculation by Larsen *et al.* from a luminescence study that · OH radicals are generated in thrombin-activated platelets.[5]

With stronger stimulants, such as thrombin and the calcium ionophore ionomycin, the ESR signals due to DMPO-OH were stronger than those in the presence of a weaker stimulant ADP (Figure 1). The generation of · OH also depended on the concentration of the stimulant: As shown for thrombin in Figure 2, the generation of · OH increased with increase in thrombin concentration to a plateau with thrombin at 4 unit/ml. This dose-dependency of the radical generation is consistent with that of platelet function, as shown in the same figure for aggregability by thrombin. Moreover, the generation of · OH was inhibited by an inhibitor of platelet activation, such as forskolin (Figure 1(e)). These results suggest that generation of · OH radicals occurs during the process of activation of platelets. We furthermore investigated the effects of phenolic antioxidants on the radical generation. As shown in Table I, · OH generation was inhibited by catechol compounds and hydroquinone.

To clarify the characteristics of the generation of · OH, we investigated the effect of the time of preincubation of the platelets with stimulants on radical generation. When platelets were activated by thrombin, the generation of · OH was greatest 1.5 min after addition of the stimulant (Figure 3), whereas when ADP was used as a stimulant, the generation of the radicals reached a maximum more quickly (data not shown). This difference may reflect a difference in the activation rates by different stimulants.[11]

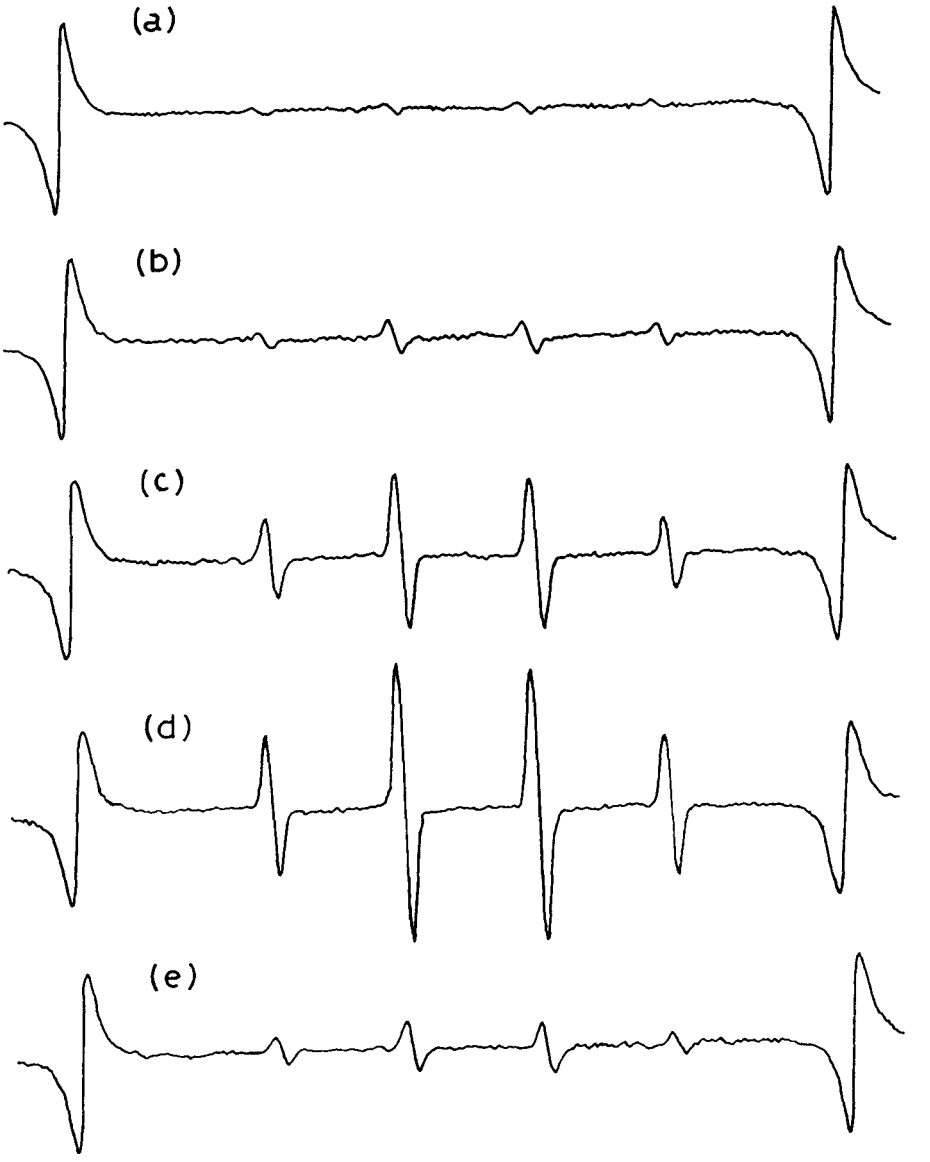

FIGURE 1 ESR spectra of active oxygen species generated in platelets with no stimulant (a), 50 μM ADP (b), 2 unit/ml thrombin (c), 4 μM ionomycin (d) and 2 unit/ml thrombin with 50 μM forskolin (e). Spectra were measured 1.5 min after addition of stimulants. Signals on either side of each ESR spectrum are the third and fourth signals ($\Delta H_{3\text{-}4}$ = 86.9 Gauss) due to standard magnanese (II) ion doped in MgO powder.

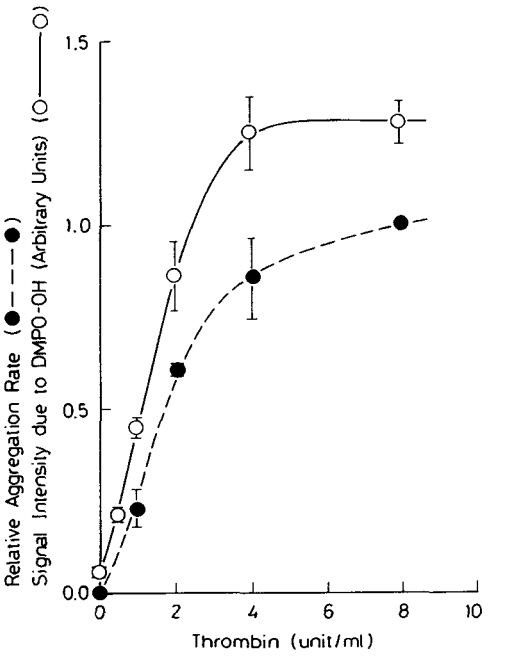

FIGURE 2 Effects of thrombin concentration on signal intensity due to DMPO-OH and aggregation rate of platelets. ESR spectra were recorded 1.5 min after addition of thrombin and signal intensities were measured. The aggregation rate with 8 unit/ml thrombin was defined as 1.0. Data are means ± S.D. for three experiments.

DISCUSSION

The present results show that · OH radicals are generated in platelets, and that their generation is accelerated by activation of the platelets. The generation of · OH seems to be carried out during some enzymatic processes which regulate platelet functions. Since active oxygen species are suggested to be generated during arachidonate metabolism[12,13] which regulates platelet activity, it is possible that · OH radicals are

TABLE I
Effects of 1 mM dihydric phenols on signal intensity due to DMPO-OH in bovine platelet suspension activated by 2 unit/ml thrombin

Compound	Percent Signal Intensity
None	100
Pyrocatechol	40.8 ± 7.0
Hydroquinone	75.6 ± 12.6
Protocatechuic acid	38.8 ± 5.8

Data are means ± S.D. for three experiments.
Signal intensities were measured after 1.5 min incubation of platelets with the phenolic compounds and thrombin.

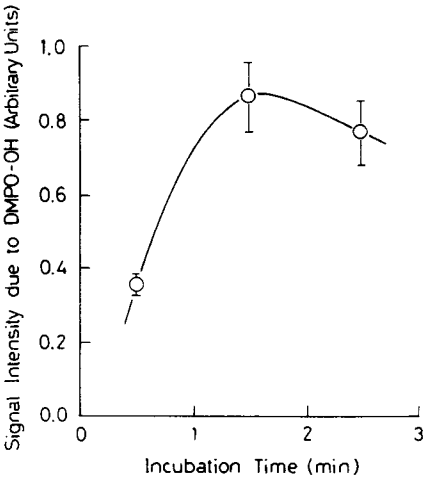

FIGURE 3 Effect of incubation time of platelets with 2 unit/ml thrombin on signal intensity due to DMPO-OH. Data are means ± S.D. for three experiments.

generated in platelets during this enzymatic process. Consistent with this idea, phenolic antioxidants, which inhibit cyclooxygenase and lipoxygenase,[14,15] markedly inhibited the generation of · OH as shown in this work. The exact mechanism of generation of · OH is still unknown, but they are likely to be formed during peroxidase-induced reductions of various hydroperoxy acids such as during conversion of PGG_2 and PGH_2.[5]

Oxygen free radicals such as O_2^- and · OH have been suggested to cause various cell injury.[16,17] Experiments *in vivo* and *in vitro* have shown that stimulation of platelets leads to injury and detachment of endothelial cells. Therefore, the results revealed here suggest that · OH radicals generated by platelets play an important role in the endothelial cell injury. Together with other released biological active materials from platelets such as thromboxane A_2 and 5-HT, these radicals may also play an essential role in the incidence of pathogenesis of allergic inflammatory disorders such as asthma.[6]

Although enhancement of platelet function by oxygen free radicals is suggested,[18] it is still unknown how · OH radicals are involved in the processes regulating platelet functions. Moreover, although the hydroxyl spin-trapped adduct DMPO-OH was observed, O_2^- formed may actually be the active oxygen species generated in platelets, because rapid conversion of the superoxide spin-trapped adduct 5,5-dimethyl-2-hydroperoxy-1-pyrrolidinyloxy (DMPO-OOH) to DMPO-OH has recently been suggested.[19,20] Further studies on this subject are in progress.

References

1. M.S. Wolin and F.L. Belloni (1985) Superoxide anion selectively attenuates catecholamine-induced contractile tension in isolated rabbit aorta. *American Journal of Physiology*, **249,** H1127–1133.
2. G.M. Rubanyi and P.M. Vanhoutte (1986) Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. *American Journal of Physiology*, **250,** H815–821.
3. M.T. Quinn, S. Parthasarathy, L.G. Fong, and D. Steinberg (1987) Oxidatively modified low density

lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proceedings of the National Academy of Sciences U.S.A.*, **84,** 2995–2998.
4. B.M. Babior, R.S. Kipnes and J.T. Curnette (1973) Biological defense mechanisms. Production by leukocytes of superoxide, a potential bactericidal agent. *Journal of Clinical Investigation*, **52,** 741–744.
5. T. Larsen, M.B. Sørensen, R. Olsen and L. Jørgensen (1989) Effect of scavengers of active oxygen species and pretreatment with acetyl-salicyclic acid on the injury to cultured endothelial cells by thrombin-stimulated platelets. *In Vitro Cellular Development Biology*, **25,** 276–282.
6. C.P. Page (1989) Platelets and asthma. *Agents Actions Suppl.* **28,** 75–84.
7. M. Schmitz-Schumann, V. De Souza, G. Menz, G.M. Hansch, U. Rother, K. Rother and Chr. Virchow (1989) Reduced production of oxygen-free radicals of platelets in aspirin-induced-asthma. *Agents Actions Suppl.*, **28,** 195–203.
8. E.G. Janzen (1980) A critical review of spin trapping in biological systems in *Free Radicals in Biology*, Vol. IV, (ed. W.A. Pryor), Academic Press, New York, pp. 115–154.
9. S. Kitagawa, Y. Hongu and F. Kametani (1982) Effects of anions on ADF-stimulated aggregation of bovine blood platelets. *Biochemical and Biophysical Research Communications*, **104,** 1371–1375.
10. S. Kitagawa, J. Endo, R. Kubo and F. Kametani (1983) Inhibitory effects of 4,4′-diisothiocyanostilbene-2,2′-disulfonate(DIDS) on the ADP-stimulated aggregation of gel-filtered bovine blood platelets. *Biochimica and Biophysical Research Communications*, **111,** 306–311.
11. S. Kitagawa, M. Ishida, K. Kotani and F. Kametani (1987) Inhibitory effects of long-chain alkyltrimethylammonium ions on aggregation of bovine platelets and the relation of their effects to Ca^{2+} mobilization. *Biochimica et Biophysica Acta*, **905,** 75–80.
12. F.A. Kuehl, Jr., J.L. Humes, R.W. Egan, E.A. Ham, G.C. Beveridge and C.G. Van Arman (1977) Role of prostaglandin endoperoxide PGG_2 in inflammatory processes. *Nature*, **265,** 170–173.
13. P. Wörner (1981) Arachidonic acid-induced chemiluminescence of human platelets: Contribution of the prostaglandin and lipoxygenase pathways. *Thrombosis Haemostasis*, **46,** 584–589.
14. K. Sekiya, H. Okuda and S. Arichi (1982) Selective inhibition of platelet lipoxygenase by esculetin. *Biochimica et Biophysica Acta* **713,** 68–72.
15. S. Kitagawa, H. Fujisawa, S. Baba and F. Kametani (1991) Inhibitory effects of catechol derivatives on arachidonic acid-induced aggregation of rabbit platelets. *Chemical and Pharmaceutical Bulletin*, **39,** 1062–1064.
16. B. Halliwell and J.M.C. Gutteridge (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, **219,** 1–14.
17. D.T.-Y. Chiu and S. Claster (1988) Measurement of red cell membrane oxidation and the generation of oxidative intermediates. In *Red cell membrane*, Methods in Hematology 19 (S.B. Shohet and N. Mohandas eds), Churchihill, Livingstone, pp. 203–236.
18. R.J. Handin, R. Karabin and G.J. Boxer (1977) Enhancement of platelet function by superoxide anion. *Journal of Clinical Investigation*, **59,** 959–965.
19. F.W. Kleinhans and S.T. Barefoot (1987) Spin trap determination of free radical burst kinetics in stimulated neutrophils. *Journal of Biological Chemistry* **262,** 12452–12457.
20. G.M. Rosen, B.E. Britigan, M.S. Cohen, S.P. Ellington and M.J. Barber (1988) Detection of phagocyte-derived free radicals with spin trapping techniques: effect of temperature and cellular metabolism *Biochimica et Biophysica Acta*, **969,** 236–241.