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LIPID PEROXIDATION IN HEMOLYSATE OF RABBIT ERYTHROCYTES

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Summary - Incubation of hemolysate of rabbit erythrocytes with Zn^{2+} induced lipid peroxidation. The combined use of superoxide dismutase and catalase and histidine inhibited the reaction but mannitol did not, suggesting that the active oxygen species, probably singlet oxygen, arising in the interaction between superoxide and hydrogen peroxide is the responsible factor. Ascorbate had biphasic effects, i.e., a promoting one at low and an inhibitory one at high concentrations, and α-dl-tocopherol also suppressed lipid peroxidation. This reaction was sensitive to high NaCl concentrations, and protein in the reaction mixture had a non-specific inhibitory action.

Production of lipid peroxide has been considered to reflect the outcome of peroxidation damage to cellular polyunsaturated fatty acids, and active oxygen species to be intimately implicated in this reaction (1-7). So far a number of studies have used erythrocytes as target cells and evaluated hemolysis and malondialdehyde formation during oxidative stress as indexes of lipid peroxidation (2, 8-12). This communication describes a method by which hemolysate of rabbit erythrocytes instead of intact cells is used to produce lipid peroxide. Some characteristics of the reaction are discussed, particularly in relation to the functions of various scavengers of active oxygen species.

Materials and methods

Superoxide dismutase (Bovine blood, 3,000u/mg protein) and catalase (Bovine liver, 30,000 Sigma units/mg protein) were purchased from Sigma Chem. Co. D-(-)-mannitol, α-dl-tocopherol, Tween 20, D-histidine, α-ascorbic acid and other reagents were products of Nakarai Chem. Co. Bovine albumin powder (Fraction V from bovine plasma) was from Armour Pharmaceutical Co. Enzymes were inactivated by autoclaving for 20 min.

Heparinized blood was drawn from male rabbits, weighing about 2 kg, which had been fed a regular diet and water ad lib. Three rabbits were used interchangeably, as all of them exhibited the same tendency with little individual variation. Following centrifugation, plasma and buffy coat were removed, eryth

Abbreviations used : HEPES, N-hydroxyethylpiperazine'N'-ethanesulfonic acid, pH 7.4; Zn-HEPES, 17mM HEPES containing 1.0mM $ZnCl_2$.

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rocytes were washed three times with saline and suspended to contain 10g of hemoglobin/100ml. They were stored in small aliquots at -80°C without any effect on subsequent determination. Before use they were thawed and treated in ice by sonication with a Heat Systems Cell Disruptor Model W-225R (Ultrasonics, Inc.) for 60 sec at 60 watts. One-tenth of a milliliter of the hemolysate was used without further centrifugation in a final reaction volume of 0.5ml of 17mM HEPES buffer containing the constituents described in each experiment.

Lipid peroxide was measured by a slight modification of the fluorometric method of Yagi (13) with thiobarbituric acid. The lipid peroxide concentration of the sample was determined in terms of malondialdehyde (n moles/g of hemoglobin) based on the values of tetraethoxypropane as a standard. α-dl-Tocopherol was prepared as an emulsion, 1 part of Tween 20 to 4 parts of α-dl-tocopherol (w/w).

Results

Incubation of hemolysate in HEPES buffer alone for 4 hours at 37°C failed to yield any further increase in lipid peroxide above the basal level, but there was an increase in undeionized water. As some contaminant metal ions were postulated to be necessary, attempts were made to induce an increase by incorporating one of the following into the reaction mixture: $FeCl_2$, $FeCl_3$, $MnCl_2$, CuCl, $CuCl_2$, $MgCl_2$, $CaCl_2$, and $ZnCl_2$ at concentrations of 10^{-3} to 10^{-7}M. Among those tested, 1.0mM $ZnCl_2$ consistently resulted in an augmentation of lipid peroxide, and there was less and inconstant enhancement by $CaCl_2$. Consequently the following determinations were made in 17mM HEPES containing 1.0mM $ZnCl_2$.

Hemolysate in Zn-HEPES containing 30.8mM NaCl was incubated at 37°C and aliquots were removed at certain intervals and assayed for lipid peroxide. Fig. 1A shows that the lipid peroxide in hemolysate increased linearly in a time-dependent manner, while that of intact cell suspension in 154mM NaCl in Zn-HEPES remained unchanged during this period of incubation. In the latter condition, the value was not affected by the presence or absence of $ZnCl_2$. In the subsequent experiments the incubation time was limited to $2\,^1/_2$ hours, which was sufficient to almost double the lipid peroxide value of the control specimen, which was kept in an ice bath for the same length of time. Hemolysate was then incubated in varying concentrations of NaCl in Zn-HEPES. As shown in Fig. 1B, production of lipid peroxide was almost completely suppressed at 92.4mM of NaCl concentration.

Both superoxide dismutase and catalase inhibited lipid peroxidation, but each autoclaved enzyme also inhibited the reaction to almost the same degree

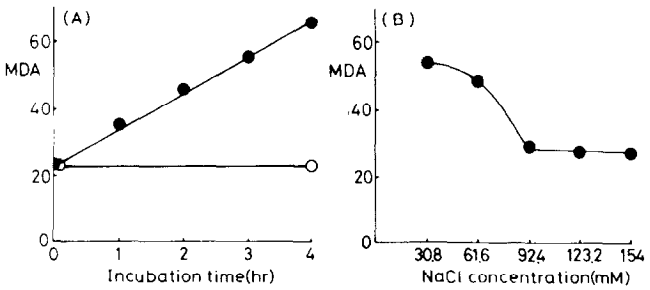

Fig. 1 Time course of lipid peroxidation of hemolysate (●) in hypotonic saline in Zn-HEPES and of intact cells in isotonic saline in Zn-HEPES (○)(A) and effect of NaCl on the reaction (B). Ordinates indicate malondialdehyde expressed in n moles/g hemoglobin. Experimental details are given in the text.

(Fig. 2). When they were combined, however, the suppressive effect was greater than when either one was used alone. The difference between native and autoclaved enzymes was more marked, indicating the specific effect of both enzymes. In order to determine whether the protein nonspecifically inhibited the reaction, bovine plasma albumin was incorporated in the reaction system and was found to behave as an inhibitor in a dose-dependent manner (data not shown). Bovine plasma albumin, in an amount adjusted to be equal to the sum of the proteins of superoxide dismutase and catalase used in this experiment, yielded approximately the same value as the autoclaved enzymes.

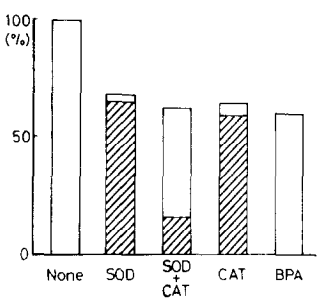

Fig. 2 Effects of superoxide dismutase (SOD), catalase (CAT) and bovine plasma albumin (BPA) on lipid peroxidation of hemolysate of rabbit erythrocytes. Ordinate indicates percent increment of the lipid peroxide value above the basal value in the hemolysate which was kept in an ice bath for the same length of time, and abscissa additions to the reaction mixture. SOD, 6,000u (2mg protein); catalase, 6,000 Sigma units (0.2mg protein); bovine plasma albumin, 2.2mg. Total height of the middle three columns shows the lipid peroxide levels with autoclaved enzymes and hatched portions those with native enzymes, respectively.

Table 1 Effects of ascorbate, α-dl-tocopherol, and histidine on lipid peroxidation in hemolysate of rabbit erythrocytes

Additions to complete system		Percent increment of MDA* above the basal level
None		100
Ascorbate (mM)	5	0
	1.0	18
	0.1	150
	0.01	115
α-dl-tocopherol (mM)	1.0	0 (0)**
	0.1	31 (100)
	0.01	100 (100)
Histidine (mM)	1.0	31
	0.5	51
	0.25	90

MDA* : Malondialdehyde.
** : Figures in parentheses indicate values with Tween 20 only, used for emulsification of the corresponding α-dl-tocopherol.

The effects of histidine, α-dl-tocopherol, α-ascorbic acid are shown in Table 1. Mannitol, a scavenger of hydroxyl radical, was also tested but had no demonstrable effect (data not shown). As Tween 20 itself was found to suppress lipid peroxidation in this system, the effect of Tween 20 was examined with and without α-dl-tocopherol. Ascorbic acid had both inhibitory and enhancing effects depending on the concentration.

Sonicated hemolysate was centrifuged at 35,000 x g for 60 min at 0°C to remove the cell membrane fractions, and the supernatant was similarly assayed and compared with the uncentrifuged hemolysate. When cell membranes were removed, lipid peroxide value decreased by 25%. The difference became more significant after incubation; compared with a 100% increase in the uncentrifuged specimen, the centrifuged one gave only a 32% rise above the corresponding basal level. The addition of 0.1% triton X-100 to the hemolysate decreased the basal level by 40%.

Discussion

The present study shows that hemolysate of rabbit erythrocytes induces lipid peroxidation during incubation at 37°C in the presence of Zn^{2+} at low

NaCl concentrations. Transitional metal ions, especially iron, are essential catalysts for lipid peroxidation (1, 3, 5-7, 14, 15). The absence of an increase in lipid peroxidation in HEPES buffer alone is interpreted as indicating that additional metals are required for the reaction. Among the various metal ions tested, only zinc was found to be required, and its most plausible role therefore appears to be the precipitation of hemoprotein (16), which is known to strongly inhibit peroxidation (17), thus allowing the reaction to proceed uninhibited.

The additive inhibitory effect of superoxide dismutase and catalase suggests that the active oxygen species formed in the interaction between superoxide anion and hydrogen peroxide results in the peroxidation of rabbit erythrocyte hemolysate. Moreover, the inhibitory effect of histidine, a singlet oxygen scavenger, and the apparent absence of such an effect of mannitol support the view that singlet oxygen may also be causally related. On the basis of the results of their determinations of thiobarbituric acid-reactive material and lipid hydroperoxide assays, Svinger et al.(6) defined the initiation and propagation of lipid peroxidation and showed that superoxide is most likely to be involved in the initiation, while singlet oxygen participates in the propagation process. In contrast to several reports of a loss of inhibitory activity of superoxide dismutase when heated (1, 2, 5), the present study indicates that not only heated enzyme but also bovine plasma albumin inhibits the reaction. This observation is compatible with our previous finding that not only native but also boiled enzymes and other purified proteins have non-specific inhibitory effects on nitroblue tetrazolium reduction, in which superoxide is intimately involved (18). Recently Halliwell and Gutteridge (15) have suggested that the inhibitory effect of heat-inactivated superoxide dismutase is due to released free Cu^{2+}, but it remains to be determined whether the same explanation is applicable to the persistence of activity of heated catalase and of albumin.

This reaction is also sensitive to NaCl high concentrations, which inhibit the process. This finding is in line with that of Pederson and Aust (1) that autoxidation proceeds faster at low ionic strength. Amano (19) also studied the

effect of salt concentration on nitroblue tetrazolium reduction and found its maximal rate to be at 42 to 53mM NaCl, with a decline at higher concentrations.

Ascorbate has been shown to promote lipid peroxidation in a relatively narrow range of concentrations, i.e., 0.1 to 0.66mM (3, 17, 20). Higher concentrations of ascorbate inhibit the reaction, and Wills (17) assumes that an optimum concentration is necessary to keep the appropriate ferrous/ferric ratio, which is essential for the maintenance of the autoxidation process. This dual property of ascorbate is not limited to this reaction and has been shown not only in ascorbate-induced swelling and lysis of mitochondria (21) but also in leukocyte oxygen metabolism (22, 23). Vitamin E, the action of which is potentiated by nitroblue tetrazolium, has been demonstrated to cause marked depression of the respiratory burst in normal leukocytes, to inhibit the platelet response to aggregating agents and to block the lymphocyte response to phytohemagglutinins (24). The inhibitory role of vitamin E in lipid peroxidation may thus be regarded as one of the non-specific scavenger systems for free radicals.

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References

1. Pederson, T. C., and Aust, S. D.(1972) Biochem. Biophys. Res. Commun. 48, 789-795.
2. Fee, J. A., and Teitelbaum, H. D.(1972) Biochem. Biophys. Res. Commun. 49, 150-158.
3. Pederson, T. C., and Aust, S. D.(1973) Biochem. Biophys. Res. Commun. 52, 1071-1078.
4. Zimmermann, R., Flohé, L., Weser, U., and Hartmann, H-J.(1973) FEBS Lett. 29, 117-120.
5. Gutteridge, J. M. C.(1977) Biochem. Biophys. Res. Commun. 77, 379-386.
6. Svingen, B. A., O'Neal, F. O., and Aust, S. D.(1978) Photochem. Photobiol. 28, 803-809.
7. Nakano, K., and Obo, F.(1979) Biochem. Biophys. Res. Commun. 89, 1239-1244.
8. Dodge, J. T., Cohen, G., Kayden, H. J., and Phillips, G. B.(1967) J. Clin. Invest. 46, 357-368.
9. Mengel, C. E., Kann, H. E., Jr., and Meriwether, W. D.(1967) J. Clin. Invest. 46, 1715-1723.
10. Stocks, J., and Dormandy, T. L.(1971) Brit. J. Haematol. 20, 95-111.
11. Fee, J. A., Bergamini, R., and Briggs, R. G.(1975) Arch. Biochem. Biophys. 169, 160-167.
12. Vertongen, F., Heyder-Bruckner, C., Fondu, P., and Mandelbaum, I.(1981) Clin. Chim. Acta 116, 217-222.
13. Yagi, K.(1976) Biochem. Med. 15, 212-216.

14. Talcott, R. E., Shu, H., and Wei, E. T.(1979) Biochem. Pharmacol. 28, 665-671.
15. Halliwell, B., and Gutteridge, J. M. C.(1981) FEBS Lett. 128, 347-352.
16. Oelshlegel, F. J., Jr., Brewer, G. J., Knutsen, C., Prasad, A. S., and Schoomaker, E. B.(1974) Arch. Biochem. Biophys. 163, 742-748.
17. Wills, E. D.(1969) Biochem. J. 113, 315-324.
18. Amano, D., Kagosaki, Y., Usui, T., Yamamoto, S., and Hayaishi, O.(1975) Biochem. Biophys. Res. Commun. 66, 272-279.
19. Amano, D.(1976) Ann. Paed. Jap. 22, 99-107.
20. Gibson, D. D., Hornbrook, K. R., and McCay, P. B.(1980) Biochim. Biophys. Acta 620, 572-582.
21. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L., and Smith, E.(1964) J. Biol. Chem. 239, 604-613.
22. Bigley, R., Gerhardt, N. B., Eigner, T., Dehlinger, A., Niedra, J., and Stankova, L.(1978) J. Reticuloendothel. Soc. 24, 1-7.
23. Kraut, E. H., Metz, E. N., and Sagone, A. L., Jr.(1980) J. Reticuloendothel. Soc. 27, 359-366.
24. Repine, J. E., Rao, G., Beall, G. D., and White, J. G.(1978) Am. J. Pathol. 90, 659-674.