Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 474-480

SUPEROXIDE AND HYDROGEN PEROXIDE-DEPENDENT LIPID PEROXIDATION IN INTACT AND TRITON-DISPERSED ERYTHROCYTE MEMBRANES

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Received November 28, 1983

SUMMARY. Isolated erythrocyte membranes incubated with xanthine, xanthine oxidase, and Fe(III) underwent lipid peroxidation, as indicated by the thiobarbituric acid reaction and iodometric determination of hydroperoxides. In detergent-free medium (phosphate buffered saline) peroxidation was inhibited by superoxide dismutase, catalase, and EDTA; but was promoted by OH· scavangers, eg. mannitol. Generation of OH· in the system via iron-catalyzed reduction of H_2O_2 by O_2^- was demonstrated by EPR spectrometry using spin trapping. In membranes treated with Triton X-100 lipid peroxidation was stimulated by EDTA and suppressed by OH· traps. This and other evidence suggests that OH· in the medium was an effective initiator of lipid peroxidation in detergent-dispersed membranes, but not in intact membranes.

Metabolic reduction of molecular oxygen to species such as O_2^- and H_2O_2 is believed to be a crucial event in many forms of oxygen toxicity (1). Individually, O_2^- and H_2O_2 react relatively slowly with biological targets such as amino acid residues and unsaturated lipids. However, in the presence of ligated metal ions that undergo redox cycling, these species can interact to produce OH·, a strong oxidant (2,3). In the case of chelated iron, this process, the so-called Haber-Weiss cycle [O_2^- + Fe(III) → Fe(II) + O_2; H_2O_2 + Fe(II) → OH^- + OH· + Fe(III)] has been implicated as the initiating step in lipid peroxidation, a widely studied form of oxidative damage in cell membranes (4,5,6). However, tests for OH· involvement in lipid peroxidation and other cytotoxic effects induced by O_2^- and H_2O_2 have not always been conclusive. For example, a system has been described (6) in which lipid peroxidation was inhibited by SOD, but not

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Abbreviations: SOD, superoxide dismutase; RBC, human red blood cell; PBS, 125 mM NaCl, 25 mM sodium phosphate (pH 7.4); EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; TBA, 2-thiobarbituric acid; EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OH, 5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl.

0006-291X/84 $1.50

by catalase. Also, there have been conflicting reports on the effects of OH· scavenging agents. In some cases (5,6,7) large protective effects were observed, while in others (6,8,9) no protection was seen. In the present study, we ask how the physical state of peroxidizable membrane lipids may influence conclusions about reaction mechanism. The test system is the RBC ghost exposed to xanthine oxidase plus xanthine as a source of O_2^- and H_2O_2. Determinants of lipid peroxidation in intact ghosts and ghosts dispersed by the non-ionic detergent Triton X-100 are compared.

MATERIALS AND METHODS. Xanthine oxidase (Grade III, 1.3 U/mg), catalase (18,000 U/mg), and SOD (2900 U/mg) were obtained from Sigma. Any SOD in catalase was removed by gel filtration on Sephadex G-75. Before use, xanthine oxidase (∿1 U/ml in terms of urate formation (10)) was dialyzed against 50 mM choline, 0.01 mM EDTA, 10 mM phosphate (pH 7.4). Triton X-100, EDTA, and DTPA were from Sigma. Desferrioxamine (Desferal) was provided by Ciba-Geigy. All other chemicals were reagent grade and all solutions were prepared with deionized, glass distilled water.

Freshly drawn human blood was obtained from the Blood Center of Southeastern Wisconsin. RBC membranes (white ghosts, essentially hemoglobin-free) were washed and concentrated by tangential-flow filtration in a Millipore-Pellicon Cassette System (11) and used within one week. Membrane protein was determined according to Lowry *et al* (12) using serum albumin as the standard. Membrane suspensions in PBS were incubated with xanthine, xanthine oxidase, and $FeCl_3$ to initiate lipid peroxidation. Unless indicated otherwise, reactions were carried out at 37°C in Stirrer-Bath vessels (Yellow Springs Instruments). Peroxidation was assessed by reacting aldehyde by-products with TBA and by direct iodometric determination of lipid hydroperoxides. At different time points samples for TBA assay were mixed with 0.15% SDS/0.15% butylated hydroxytoluene and reacted with TBA as described previously (13). Absorbance readings at 532 nm were corrected for zero-time values, which were in the 0.01-0.02 range. The iodometric assay was an adaptation of previous approaches (14,15). A 0.5 ml sample (mixed with 0.1 mM EDTA to prevent peroxide decomposition) was vortexed with 0.8 ml of $CHCl_3$-CH_3OH (2:1). The organic phase, 0.4 ml, was evaporated to dryness under N_2 in a microcentrifuge tube, followed by the addition of 0.3 ml of N_2-sparged CH_3COOH-$CHCl_3$ (3:2) and 0.02 ml of 6 M KI. After 5 min in the dark at 25°C, the solution was mixed with 0.9 ml of 20 mM cadmium acetate, centrifuged, and the absorbance of the supernate at 353 nm was recorded. All readings were corrected for blanks that lacked membranes. Hydroperoxide levels were calculated using an extinction coefficient of 21,900 ± 300 $M^{-1}cm^{-1}$, which was determined with standard cumene hydroperoxide.

EPR experiments were carried out at 25°C using a Varian E-109 spectrometer operating at 9.5 GHz and utilizing 100 kHz field modulation. A TM cavity was used to improve the signal to noise ratio of the aqueous samples. Hydroxyl radical was detected by spin trapping with DMPO to produce DMPO-OH (16). Samples were mixed outside the cavity, then rapidly aspirated (< 15 sec) into an aqueous flat cell for EPR measurements. The initial rate of DMPO-OH formation was determined by measuring the amplitude change of a center hyperfine line (relative intensity 2) of the approximately 1:2:2:1 spectrum, and referring this change to the amplitude of one of the hyperfine lines of standard 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy.

RESULTS. Incubation of RBC ghosts in the presence of xanthine, xanthine oxidase, and Fe(III) resulted in progressive peroxidation of polyunsaturated

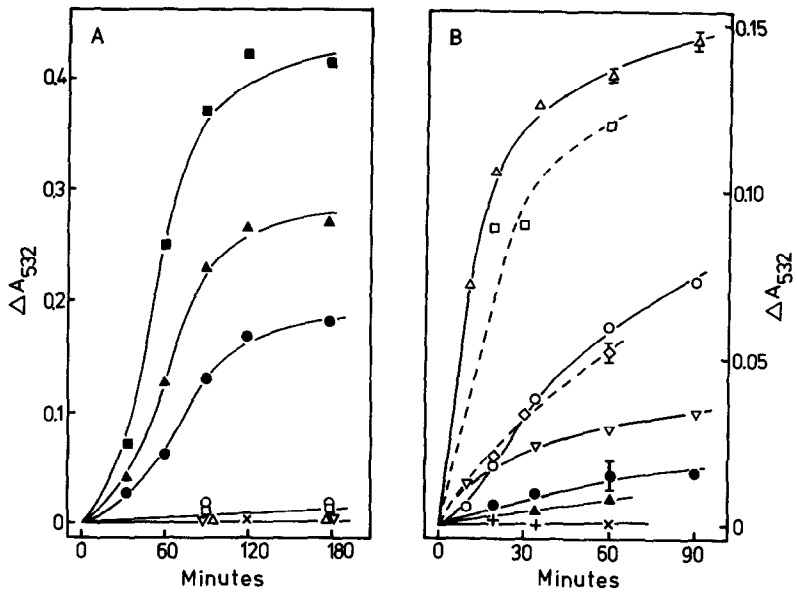

Fig. 1. Formation of thiobarbituric acid-reactive products. Membranes (1 mg protein/ml) in PBS were incubated in the presence of 1 mM xanthine, 0.01 U xanthine oxidase/ml, 0.1 mM NaN_3, and 0.05 mM $FeCl_3$. Azide was included to inhibit any endogenous catalase (omitted when catalase was added). A, reactions in the absence of detergent. Additional components in reaction mixtures: (●) none; (▲) ethanol (50 mM); (■) mannitol (50 mM); (○) catalase (50 µg/ml); (□) SOD (50 µg/ml); (△) EDTA (0.1 mM); (▽) DTPA (0.1 mM). B, reactions in the presence of Triton X-100 (0.2%, v/v). Additional components: (○) none; (△) EDTA (0.1 mM); (□) EDTA (0.1 mM), mannitol (10 mM); (◇) EDTA (0.1 mM), mannitol (100 mM); (▽) DTPA (0.1 mM); (●) catalase (50 µg/ml); (▲) SOD (50 µg/ml); (+) desferrioxamine (0.1 mM). Controls (×) lacked xanthine oxidase. Points with error bars indicate averages ± deviations of duplicate experiments. (A Triton blank without membranes produced no detectable TBA reactivity).

lipids, as indicated by the increase in 532 nm absorbance of TBA adducts (Fig. 1A). No reaction was observed when xanthine oxidase was omitted from the reaction mixture, and catalytic amounts of SOD or catalase inhibited strongly, indicating that both O_2^- and H_2O_2 are required. When chelators were present in 2-fold excess over added Fe(III), eg. EDTA, DTPA, or desferrioxamine (not shown), no peroxidation occurred, presumably because iron was removed from the membranes. The possibility that lipid peroxidation is triggered by OH• generated via the iron-catalyzed Haber-Weiss reaction was checked by introducing OH• traps into the system. As shown in Fig. 1A, mannitol or ethanol in great excess over membrane lipids (eg. the phospholipids were ∿0.7 mM in the bulk suspension) not only failed to inhibit TBA-detectable peroxidation, but actually enhanced the reaction. Consistent results were obtained when whole ghosts treated with xanthine/xanthine oxidase/iron were analyzed for

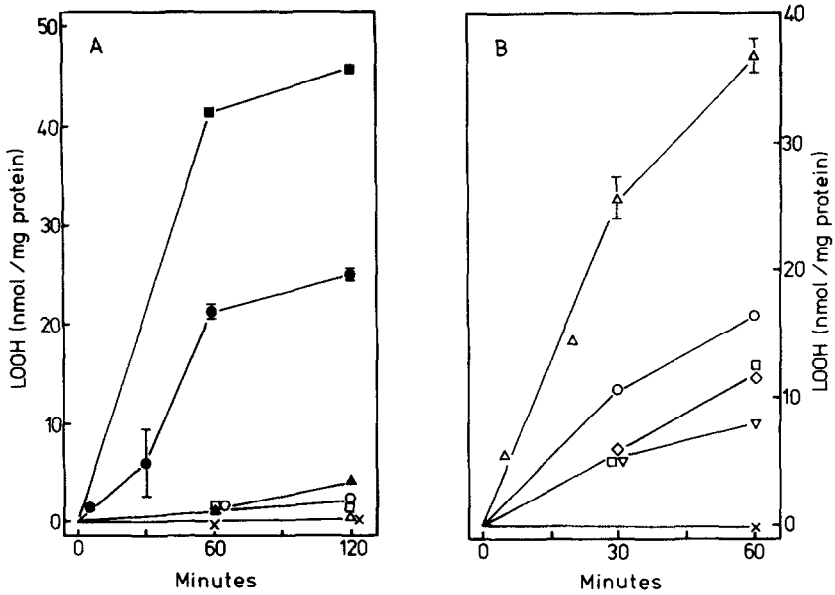

Fig. 2. Formation of lipid hydroperoxides (LOOH). The reaction system consisted of membranes (1 mg protein/ml in PBS), xanthine (1 mM), xanthine oxidase (0.01 U/ml), NaN_3 (0.1 mM, except when catalase added), and $FeCl_3$ (0.1 mM). A, reactions in the absence of detergent. Other components in reaction mixtures are as follows: (●) none; (○) SOD (50 μg/ml); (□) catalase (50 μg/ml); (△) EDTA (0.2 mM); (■) mannitol (50 mM). Also shown is a control without added iron (▲). B, reactions in the presence of Triton X-100 (0.2%, v/v). Other additions: (○) none; (△) EDTA (0.2 mM); (▽) DTPA (0.2 mM); (□) EDTA (0.2 mM), ethanol (50 mM); (◇) EDTA (0.2 mM), mannitol (50 mM). Average values ± SD (n=3) are shown for some experiments. Controls lacking xanthine oxidase are indicated (×).

hydroperoxide levels. As shown in Fig. 2A, the rate of hydroperoxide formation was relatively low without added Fe(III) and is attributed to background metal ion in the membranes and reagents. Addition of 100 μM $FeCl_3$ resulted in a large stimulation of the reaction, which subsided after 1 h. Catalase, SOD, and EDTA all strongly inhibited peroxide formation, but mannitol acted in a stimulatory fashion, as shown with the TBA assay.

The time course of TBA adduct formation in Triton X-100-dispersed ghosts is shown in Fig. 1B. Note that the initial rate at which A_{532} increased during xanthine/xanthine oxidase/iron-treatment is similar to that observed for intact ghosts. However, the responses to OH· scavengers and certain chelators were strikingly different. For example, in the Triton-containing system, EDTA greatly stimulated the reaction (probably by stabilizing Fe(III) against hydration (17)), while mannitol (10 mM and 100 mM) in the presence of EDTA inhibited in a dose-dependent manner. (Similar results were obtained using

Lubrol as the detergent.) In contrast to EDTA, DTPA suppressed peroxidation, but not to as great an extent as desferrioxamine. DTPA and desferrioxamine are known to hinder the reduction of Fe(III) by O_2^- (17,18). Catalase and SOD acted protectively, thereby confirming the requirement for O_2^- and H_2O_2. In separate experiments the TBA results were checked by tracking the formation of lipid hydroperoxides (Fig. 2B). As can be seen, the initial rate of EDTA-stimulated peroxidation was strongly inhibited by ethanol or mannitol and once again DTPA, unlike EDTA, suppressed the basal reaction.

Using DMPO as an extramembranous spin trap for OH$^\bullet$ we obtained unambiguous evidence for the formation of this radical in our reactions (Table I). Generation of spin adduct DMPO-OH in the absence of detergent was strongly enhanced by EDTA (cf. Expts. 1 and 3), and in the presence of this chelator

TABLE I

Hydroxyl spin adduct formation in membrane suspensions incubated with xanthine/xanthine oxidase/iron in the absence and presence of Triton X-100

	DMPO-OH (nmol/min)[a]	
Expt.	- Triton	+ Triton
1. Complete	0.51 ± 0.01[b] (100)	0.38 ± 0.01[b] (100)
2. - ghosts	0.49 (96)	ND
3. - EDTA	0.05 (10)	ND
4. - EDTA + DTPA (0.2 mM)	ND[c]	0.03 ± 0.02 (8)
5. + catalase (150 U/ml)	≃0 (0)	≃0 (0)
6. + SOD (17 μg/ml)	0.12 (23)	0.08 (21)
7. + mannitol (50 mM)	0.33 (64)	ND
8. + mannitol (100 mM)	0.19 (37)	0.11 (29)

[a] Initial rate of formation of 5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl radical (DMPO-OH). The complete reaction mixture consisted of ghosts (1 mg protein/ml in PBS), xanthine (1 mM), xanthine oxidase (0.01 U/ml), NaN_3 (0.1 mM, except for Expt. 5), EDTA (0.2 mM), $FeCl_3$ (0.1 mM), and DMPO (30 mM). Where indicated, Triton X-100 (0.2%, ∿3 mM) was included. Omitted (-) or added (+) components in different experiments are indicated.

[b] Average ± deviation of values from duplicate experiments. Numbers in parentheses are percentages.

[c] Not done.

membranes had virtually no effect on the reaction rate (cf. Expts. 1 and 2). The observed inhibitory effects of mannitol, SOD, and catalase are consistent with the involvement of OH• originating from the iron/Haber-Weiss reaction. It is apparent, however, that OH• reacting with DMPO in the aqueous compartment was not important in triggering lipid peroxidation, since mannitol affected these processes in diametrically opposite ways, attenuating DMPO-OH formation while stimulating peroxidation. Spin adduct was also detected in the presence of Triton (Table I), although its rate of formation was somewhat lower.

DISCUSSION. We have shown that lipid peroxidation is affected differently by certain chelators and inhibitors when whole ghosts are compared with Triton-dispersed ghosts. The implication is that physical state of membrane lipids should be taken into account when these agents are used for gaining information about reaction mechanism.

Our results using intact membranes (Figs. 1A, 2A; Table I) are consistent with the following scenario. Because of its short lifetime OH• generated in the medium collides infrequently with membranes and the rate of lipid peroxidation is low. However, longer-lived O_2^- and H_2O_2 migrate to the membrane surface where, in the absence of external chelators, redox cycling of bound iron results in the formation of a highly oxidizing species, presumably OH. Encountering substrates in exceedingly high local concentrations, this species reacts so rapidly that interception by exogenous traps is virtually impossible. Non-protection by OH• scavengers has been described (6,8) and in some instances has been used as evidence against OH• intermediacy. If anything, we found that scavengers stimulated peroxidation in intact ghosts, a possible explanation being that xanthine oxidase was protected against auto-inactivation (8). A similar effect was observed in connection with O_2^-/H_2O_2-mediated lysis of resealed ghosts (19).

Dispersing membrane lipids with Triton X-100 "normalized" their oxidation behavior with respect to OH• diagnostics (Figs. 2A,2B). Under these conditions, mannitol, eg., was strongly inhibitory, evidently because competition with lipids for OH• was now possible. Detergent treatment appeared to increase the

probability of collisions between peroxidizable lipids and OH· in tne medium. (Whether free radicals of Triton, a potential OH· acceptor, mediated any of the peroxidation is not known.) In previous studies on O_2^-/H_2O_2-induced peroxidation in detergent-solubilized lipids, initiation was attributed to OH·, at least for the Fe(III)-EDTA stimulated reaction (5,6). However, direct comparisons with the behavior of intact lipid membranes have not been described.

There is a growing awareness that O_2^-/H_2O_2-mediated damage in biomolecules that bind redox metals like Fe(III) or Cu(II) may involve a site-directed attack similar to that suggested here for the RBC membrane (20). In these cases the ultimate localized oxidant might be identical to a species detected in the bulk medium, viz. OH·, but this remains to be established.

ACKNOWLEDGEMENTS. This work was supported by NSF Grant PCM-8119286. We are grateful to Drs. B. Kalyanaraman and R. Sealy of the National Biomedical ESR Center for the spin trapping measurements and for many helpful discussions.

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