Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 279-285

THIOL-DEPENDENT LIPID PEROXIDATION

Ming Tien, John R. Bucher, and Steven D. Aust

Department of Biochemistry

Michigan State University

East Lansing, Michigan 48824

Received May 26, 1982

Initiation of lipid peroxidation in liposomes by cysteine, glutathione, or dithiothreitol required iron, and was not inhibited by superoxide dismutase. The absence of superoxide involvement in thiol autoxidation was confirmed by the inability of superoxide dismutase to inhibit thiol reduction of cytochrome c. Furthermore, the rate of cytochrome c reduction by thiols was not decreased under anaerobic conditions. We suggest that lipid peroxidation initiated by thiols and iron occurs via direct reduction of iron. Control of cellular thiol autoxidation, and reactions occurring as a consequence, such as lipid peroxidation, must therefore involve chelation of transition metals to control their redox reactions.

INTRODUCTION

Amid the literature concerning the protective role of GSH and related thiols against lipid peroxidation (1-3), there are reports that indicate thiols can also act as pro-oxidants (4). Misra (5) has reported that thiols can autoxidize to produce superoxide ($O_2^{\cdot-}$) and, in the presence of iron, generate the hydroxyl radical through an iron-catalyzed Haber-Weiss reaction as shown below:

$$RSH + Fe^{3+} \longrightarrow RS + Fe^{2+}, \quad (1)$$

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\cdot-}, \quad (2)$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2, \quad (3)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + {}^-OH. \quad (4)$$

The hydroxyl radical is a strong oxidant capable of initiating lipid peroxidation (6). More recently, Rowley and Halliwell (4) also reported that thiols, at low concentrations, can promote iron-dependent hydroxyl radical formation via a similar mechanism as proposed by Misra. However, these workers proposed that the reduction of Fe^{3+} by thiols (reaction 1) involved a $O_2^{\cdot-}$ intermediate, and that thiol-dependent reduction of Fe^{3+}, and consequent radical formation (reaction 4) can be inhibited by superoxide dismutase (SOD).

0006-291X/82/130279-07$01.00/0

These findings disagreed with results in our laboratory which indicated that SOD did not inhibit the initiation of lipid peroxidation dependent upon the iron-catalyzed autoxidation of several physiological and synthetic thiol compounds.

The mechanism of thiol-dependent hydroxyl radical formation also has relevance to the physiological role of SOD. While SOD has been shown to effectively inhibit radical reactions dependent on $O_2^{\cdot-}$, Fee (7) asserts that in circumstances where Fe^{3+} is directly reduced, reactions such as lipid peroxidation should not be inhibited by SOD. The significance of SOD in protection from in vivo radical damage is therefore questioned since Fee contends that the concentration of certain cellular reductants would exceed the concentration of $O_2^{\cdot-}$. In light of this controversy and the conflicting reports of SOD inhibition of thiol-dependent hydroxyl radical formation, this study further examines the involvement of $O_2^{\cdot-}$ in thiol autoxidation and in thiol-dependent lipid peroxidation.

MATERIALS AND METHODS

Reagents: Superoxide dismutase (EC 1.15.1.1.) [2,900 units/mg, measured by method of McCord and Fridovich (8)], xanthine oxidase (EC 1.2.3.2.) [0.9 units/mg, measured by cytochrome c reduction (8)], cytochrome c (type VI), xanthine, thiobarbituric acid, ADP, GSH, Cys, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Catalase (EC 1.11.1.6.) [30,000 units/mg, as measured by Holmes and Masters (9)] was thymol-free and obtained from Millipore (Freehold, NJ). All buffers were passed through Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA) to free them of contaminants. Thiol solutions were prepared in argon-purged buffers. Iron chelate solutions were prepared as described earlier (6).

Reaction Mixtures: Liposomes were made from extracted rat liver microsomal lipid (10). Rates of lipid peroxidation were assessed by the rate of malondialdehyde formation (11). All rates were determined from the initial velocity of the reaction. The data shown are results of representative experiments. Lipid peroxidation reaction mixtures were constituted in metabolic shaking water baths at 37°C under an air atmosphere. Cytochrome c reduction was monitored by the increase in 550 nm absorbance. These incubations were carried out in a cuvette in a Cary 219 spectrophotometer at 37°C. Anaerobic reactions were achieved by the use of Thunberg cells. Prior to the reaction, the cells were evacuated and flushed with argon four times. The argon was deoxygenated by the method of Vogel (12).

RESULTS

The incubation of thiols with ADP-chelated iron (ADP-Fe^{3+}) caused the peroxidation of liposomes of extracted microsomal phospholipids. Figure 1 shows the effect of thiol concentration on the rate of lipid peroxidation with added iron (0.1 mM ADP-Fe^{3+}). No peroxidation ($<$0.01 nmol MDA/min/ml) occurred in the absence of iron. Lipid peroxidation was initiated by low concentrations of Cys and maximal activity was observed at 0.25 mM Cys. The rate of peroxidation decreased at higher Cys concentrations. A similar lipid peroxidation profile was observed with dithiolthreitol but

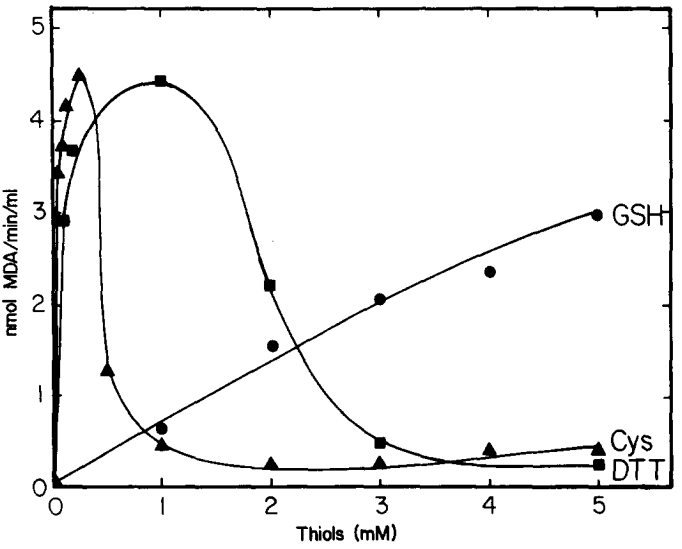

Figure 1 Effect of Thiol Concentration on Lipid Peroxidation

Incubations contained liposomes (1 umol lipid phosphate/ml), ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$) and the indicated thiols at various concentrations in 30 mM NaCl, pH 7.0 and 37°C. Reactions were initiated by the addition of thiols and assayed periodically for malondialdehyde (MDA) formation as described under "Materials and Methods".

maximal peroxidation activity occurred at 1 mM (Figure 1). GSH (1 to 5 mM) also initiated peroxidation in the presence of iron (Figure 1). However, in contrast to Cys and dithiothreitol, the rates of peroxidation increased with higher concentrations of GSH.

If the reduction of ADP-Fe^{3+} by thiols was via $O_2^{\cdot-}$, formed during thiol autoxidation, then SOD should inhibit peroxidation. As shown in Table I, SOD (10 or 50 units/ml) had little if any effect on lipid peroxidation dependent on Cys (0.08 mM), dithiothreitol (0.1 mM), or GSH (5 mM), but 10 unit/ml SOD was an effective inhibitor of lipid peroxidation dependent on the $O_2^{\cdot-}$ generating system of xanthine and xanthine oxidase.

The results obtained with thiol-dependent lipid peroxidation suggested that initiation of peroxidation by thiols does not occur via $O_2^{\cdot-}$ formation from thiol autoxidation. These results were further confirmed by examining the sensitivity of thiol reduction of cytochrome c to inhibition by SOD. Table II shows the rate of cytochrome c reduction by 0.1 mM Cys, 1 mM GSH, or 0.5 mM dithiothreitol. The thiol concentrations were adjusted such that fairly similar rates of cytochrome c reduction were obtained. Concentrations of SOD which were capable of completely inhibiting cytochrome c reduction by xanthine oxidase (Table II) had no effect on cytochrome c reduction by thiols. Additional proof that

TABLE I - EFFECT OF SUPEROXIDE DISMUTASE ON THIOL-DEPENDENT LIPID PEROXIDATION

Incubation[a] Conditions		Malondialdehyde[b] (nmol/min/ml)
A.	ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$)	0.01
B.	0.08 mM Cys, ADP-Fe^{3+}	2.84
	plus 10 unit/ml SOD[c]	2.76
	plus 50 unit/ml SOD	2.76
C.	0.1 mM Dithiothreitol, ADP-Fe^{3+}	2.81
	plus 10 unit/ml SOD	2.72
	plus 50 unit/ml SOD	2.71
D.	5 mM GSH, ADP-Fe^{3+}	2.68
	plus 10 unit/ml SOD	2.66
	plus 50 unit/ml SOD	2.77
E.	0.01 unit/ml Xanthine Oxidase, 0.33 mM Xanthine, ADP-Fe^{3+}	0.84
	plus 10 unit/ml SOD	0.04

[a]All incubations were performed with liposomes (1 umol lipid phosphate/ml) in 30 mM NaCl, pH 7.0.

[b]Rates of lipid peroxidation were assessed by measuring the malondialdehyde content of the reaction mixture at various times as described under "Materials and Methods".

little or no $O_2^{\cdot}$ is produced from the autoxidation of thiols was provided by demonstrating that the reduction of cytochrome c by thiols under anaerobic conditions equaled the rates observed with air-saturated solutions (Table II).

The reduction of cytochrome c by thiols could, however, be inhibited by EDTA (Table II). The amount of EDTA required to cause significant inhibition of cytochrome c reduction by Cys and dithiothreitol was much greater than that for GSH. The addition of

TABLE II - REDUCTION OF CYTOCHROME C BY THIOLS AND XANTHINE OXIDASE

Additions[a]	Cys (0.1 mM)	GSH (1 mM)	DTT[b] (0.5 mM)	Xanthine[c] Oxidase
None	100% (4.33)	100% (2.76)	100% (10.5)	100% (11.2)
None, Anaerobic	100%	100%	95%	3%
5 unit/ml SOD	94%	98%	86%	14%
10 unit/ml SOD	98%	91%	87%	7%
50 unit/ml SOD	85%	93%	95%	0%
1 mM EDTA	13%	0%	39%	98%

[a]Incubation mixtures contained cytochrome c (1 mg/ml) with the indicated thiol or xanthine oxidase in 0.1 mM $NaHPO_4$, pH 7.0. The rate of cytochrome c reduction was assayed as described under "Materials and Methods". The effect of various additions on the rate of cytochrome c reduction is expressed as the percentage of activity observed with no additions. Values in parenthesis represent nmol cytochrome c reduced/min/ml in the control incubations.

[b]DTT = Dithiothreitol.

[c]Incubations containing xanthine oxidase (12.5 μg/ml) also contained 0.33 mM xanthine.

either $CuCl_2$ or $FeCl_3$ could reverse the inhibition caused by EDTA. These results indicate that contaminating transition metals are likely involved in thiol reduction of cytochrome c. Experiments where cytochrome c was incubated with EDTA prior to gel filtration with Sephadex G-25 gave a preparation of cytochrome c that had a markedly lowered rate of reduction by thiols but the rate of reduction by xanthine oxidase and xanthine was not affected.

DISCUSSION

The reduction of iron plays a central role in lipid peroxidation. It is through the autoxidation reactions of reduced iron that strong oxidants are generated which can initiate lipid peroxidation (13,14). Since $O_2^{\cdot-}$ is thought incapable of directly initiating lipid peroxidation, its facilitation of these processes has been attributed to its ability to reduce iron (7). SOD will inhibit the reduction of iron by $O_2^{\cdot-}$, and its physiological role has been proposed to protect against $O_2^{\cdot-}$-induced free radical damage (15). However, other cellular reducing agents such as thiols are also capable of reducing iron (16). Due to the relatively high concentration of cellular thiols [GSH reported 3 mM or higher (17)], and the ability of these thiols to reduce iron (16,18), Fee (7) has questioned the physiological role of SOD. This controversy has recently been complicated by the claim of Rowley and Halliwell (5) that the reduction of iron by thiols occurs through a $O_2^{\cdot-}$ intermediate and that SOD can block this reduction.

The present study has demonstrated that GSH, Cys, and a synthetic thiol, dithiothreitol, were all capable of initiating lipid peroxidation. The incubation of low concentrations of Cys or dithiothreitol with ADP-Fe^{3+} effectively initiated the peroxidation of liposomes. However, at higher concentrations, lipid peroxidation activity decreased. It is possible that these two thiols act as antioxidants at higher concentrations, a proposal consistant with the findings of Rowley and Halliwell (4). In contrast, increased concentrations of GSH resulted in increased rates of peroxidation. The ability of these thiols to initiate lipid peroxidation is presumably through their capacity to reduce iron. The mechanism of this reduction does not appear to be via $O_2^{\cdot-}$ formation during thiol autoxidation since SOD had no effect of the rates of peroxidation, or on cytochrome c reduction. In agreement with previous work (5,19), cytochrome c

reduction by thiols was inhibited by EDTA and stimulated by Cu^{2+} and Fe^{3+} suggesting that transition metals are required.

Misra (5) reported that transition metals induced the autoxidation of thiols (reaction 1). He demonstrated that at alkaline pH, $O_2^{\cdot-}$ is a product of this autoxidation (reaction 2) but that $O_2^{\cdot-}$ production was very low at neutral pH. Our results suggest that at neutral pH, cytochrome c is a much better oxidant for thiols (or thiol-metal complexes) than is molecular oxygen. However, since thiols and ADP-Fe^{3+} initiate lipid peroxidation, the reduction of oxygen by thiols and transition metals must occur at neutral pH. We propose that the formation of $O_2^{\cdot-}$ could only occur through initial reduction of ADP-Fe^{3+} by thiols followed by autoxidation of ADP-Fe^{2+} (reaction 2). With regard to generation of a strong oxidant, such as the hydroxyl radical (or an iron bound oxidant of equal potential) (reactions 1-4), the effect of SOD would be to increase the rate of reaction 3. It is, however, possible that H_2O_2 is produced directly by Fe^{2+} autoxidation without a $O_2^{\cdot-}$ intermediate:

$$2Fe^{2+} + O_2 + 2H^+ \longrightarrow 2Fe^{3+} + H_2O_2. \qquad (5)$$

Consequently, in agreement with Fee (7), SOD was found to have a minimal effect on the rate of radical formation in systems where iron is directly reduced by thiols. As to the results of Rowley and Halliwell (4), we have no explanation in their findings, however, it is unclear how $O_2^{\cdot-}$ can be a necessary intermediate in the reduction of iron by thiols when iron can induce the oxidation of thiols.

It is not our intent to claim that thiols are pro-oxidants in the cell since these in vitro studies are devoid of glutathione peroxidase, an integral part of the GSH protection system (1-3). The results obtained with this model system of lipid peroxidation utilizing thiols demonstrate that in systems where iron is directly reduced, SOD has a minimal role in the prevention of radical damage. Furthermore, in light of the high cellular GSH concentration and the ability of chelation of iron to affect lipid peroxidation (20), these results also indicate that chelation or sequestering of cellular iron plays a central role in control of in vivo lipid peroxidation, and perhaps other potentially damaging effects of iron autoxidation.

ACKNOWLEDGEMENTS

We would like to express our appreciation to Mrs. Cathy M. Custer for secretarial assistance in the preparation of this manuscript. We would also like to thank Lee A.

Morehouse for his helpful discussions. Supported in part by a grant from the National Science Foundation, Grant No. PCM-7915328. Michigan Agricultural Experiment Station Journal Article Number 10440.

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