Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 361-367

THE SHORT-CHAIN HOMOLOGUE OF DIHYDROLIPOIC ACID, TETRANORDIHYDROLIPOATE, PROTECTS AGAINST IRON-INDUCED LIPID PEROXIDATION IN THE AQUEOUS PHASE

Teruyuki Kawabata*, Soon-Chee Yap† and Lester Packer*[1]

*Membrane Bioenergetics Group, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

†Palm Oil Institute of Malaysia (PORIM), Kuala Lumpur, Malaysia

Received January 3, 1994

Summary: Because iron is involved in catalysis of many biological oxidations, it is important to investigate new and novel antioxidants in terms of their effect on iron-catalyzed oxidations. We investigated the effect of dihydrolipoic acid (6,8-dimercaptooctanoic acid (DHLA)), its homologues (4,6-dimercaptohexanoic acid (bisnorDHLA) and 2,4-dimecraptobutanoic acid (tetranorDHLA)) and methyl 6,8-dimercaptooctanoate (methylDHLA) on Fe(II)-citrate-catalyzed lipid peroxide-dependent lipid peroxidation in lipid-dispersed and liposome systems. In the lipid-dispersed system, tetranorDHLA inhibited conjugated diene formation induced by Fe(II)-citrate. In the presence of tetranorDHLA, oxygen was consumed more rapidly in the reaction mixture than in the presence of the other compounds, but the oxidation rate of Fe(II)-citrate in the reaction mixture was slower than in the presence of the other compounds. This suggests that tetranorDHLA inhibited lipid peroxidation by the oxidation of tetranorDHLA itself at the site where the lipid was oxidized. © 1994 Academic Press, Inc.

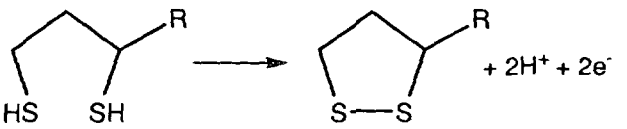
Humans are constantly exposed to many kinds of oxidative stress, both endogenous and exogenous, and it is believed that oxidative modifications of lipid, protein and nucleic acid are associated with the pathogenesis of many diseases and their complications (1-3). Transition-metal catalyzed oxidation of macromolecules is one of the most plausible and natural sources responsible for oxidative damage (4-6). In humans, iron is a factor in transition-metal catalyzed oxidative stress and may play the most important role in metal-catalyzed oxidation because of the large amounts of iron in the body and its proven catalytic activity (7-9).

When investigating a novel antioxidant, it is important to study its effect on iron-induced lipid peroxidation. Suzuki et al. (10, 11) reported on the antioxidant activity of the sulfur-containing compounds, α-lipoic acid (1,2-dithiolane-3-pentanoic acid) and dihydrolipoic acid (6,8-dimercaptooctanoic acid, DHLA) and studied the relationships between structure and antioxidant activity of DHLA and its homologues. However, in these studies iron-catalyzed oxidation was not

[1]To whom correspondence should be addressed at 251 Life Sciences Addition, Department of Molecular & Cell Biology, University of California, Berkeley, California 94720. FAX: (510)642-8313.

0006-291X/94 $5.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

used. Glutathione has been reported to work as an antioxidant in iron-overloaded mice (12); in contrast to glutathione, DHLA is a specific dithiol, which produces a dithiolane moiety (a five-membered ring structure containing disulfide bond) when it is oxidized.

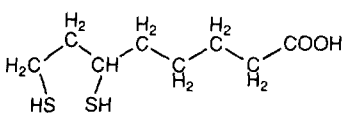


In the present study, our focus was to investigate the effect of this dithiol on iron-induced lipid peroxidation. Iron ions which have catalytic activity in oxidation are known as "free iron", distinguishing this form of iron from protein-bound iron such as transferrin and ferritin (13). Significant amounts of such ionic iron may exist in the aqueous phase as small molecular weight chelate complexes at physiological pH. In order to protect against iron-catalyzed oxidation, it may be better that antioxidants or scavengers are more hydrophilic. Hence we predicted better effects against iron-induced lipid peroxidation when we used the more hydrophilic homologues of DHLA.

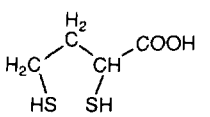
To test this hypothesis, we used short carbon chain homologues of DHLA and examined their protective effects against iron-induced lipid peroxidation. The iron ion can dramatically change its character, such as its oxidation-reduction potential and its catalytic activity, depending on the coordination environment around the iron. We selected the iron-citrate complex as an initiation species because Fe(II)-citrate is thought to be an iron chelate complex that exists *in vivo*(14).

MATERIALS AND METHODS

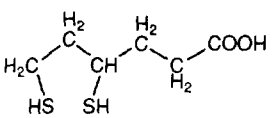
Reagents: Dihydrolipoic acid (6,8-dimercaptooctanoic acid, DHLA), bisnorlipoic acid (an oxidized form of 4,6-dimercaptohexanoic acid, bisnorDHLA), tetranorlipoic acid (an oxidized form of 2,4-dimecraptobutanoic acid, tetranorDHLA) and methyl α-lipoate (an oxidized form of methyl 6,8-dimercaptooctanoate, methylDHLA) were gifts from Asta Medica (Offenbach, Germany) (Fig. 1). Linoleic acid, N-(2-hydroxyethyl)piperazine-N'-(2-methane sulfonic acid) (Hepes), L,α-phosphatidylcholine, dioleoyl (DOPC), ferrous sulfate, pentahydrate



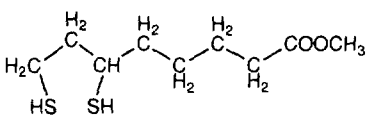
6,8-Dimercaptooctanoic acid
(DHLA)



2,4-Dimercaptobutanoic acid
(TetranorDHLA)



4,6-Dimercaptohexanoic acid
(BisnorDHLA)



Methyl 6,8-dimercaptooctanoate
(MethylDHLA)

Fig. 1. Structure of DHLA homologues and derivative which were used in the present study.

($FeSO_4 \cdot 7H_2O$), citrate (trisodium salt), sodium lauryl sulfate (SDS) were purchased from Sigma (St. Louis, MO). 2,2'-Dipyridyl was from Aldrich (Milwaukee, WI). All other reagents used were of analytical grade. BisnorDHLA, tetranorDHLA and methylDHLA were prepared by a previously described method (11) and checked by NMR spectroscopy.

DHLA and its homologues and derivative (50 mM) were dissolved in ethanol. Linoleic acid (50 mM) was dissolved in 0.5 M SDS solution, and citrate (20 mM) was in 20 mM Hepes-150 mM NaCl buffer (pH 7.4). $FeSO_4$ (20 mM) in 1.0 mM HCl was prepared just before use.

Preparation of liposomes: 2.5 ml of DOPC (25 mM) in chloroform was added to 0.25 mmoles of linoleic acid and dried by argon gas to make a thin film. 5.0 ml of Hepes-saline buffer was added to the thin film of the lipid and mixed by vortex mixer for 3 min. The milky solution was sonicated by a Biosonic III sonicator of Bronwill Scientific (Rochester, NY) for 40 min on ice. The final concentration of linoleic acid in the liposome solution was 50 mM.

Measurement of conjugated diene formation: The reaction was done at 25°C in a mixture of 0.5 mM linoleic acid, 0.4 mM citric acid and 0.2 mM $FeSO_4$ in 20 mM Hepes-saline buffer (pH 7.4) in the presence of the indicated concentration of DHLA homologues or methyl ester derivative. Linoleic acid was added to the reaction mixture as an SDS solution in the lipid-dispersed system and as a liposome suspension in the liposome system. The reaction was started by the addition of the $FeSO_4$ solution, and the absorbance at 234 nm was monitored for 1.0 min by a Perkin-Elmer spectrophotometer (Norwalk, CT). The rate of conjugated diene formation was calculated by using the molar extinction coefficient of $2.8 \times 10^4\ M^{-1} \cdot cm^{-1}$ (15).

Measurement of oxygen consumption: A Clarke-type oxygen electrode was used to measure oxygen consumption in the reaction mixture. The reaction condition was as described above. We calculated the oxygen consumption rate assuming an aqueous oxygen saturation concentration at 25°C of 253 μM.

Measurement of oxidation of Fe(II) to Fe(III): Thirty seconds after the reaction, 0.4 ml of the reaction mixture was mixed with 50 μl of 2,2'-dipyridyl (10 mM) in ethanol and 0.6 ml of Hepes-saline buffer. The absorbance at 522 nm was measured and the oxidation rate of Fe(II)-citrate was calculated from the value.

Others: DHLA homologues and the methyl derivative were measured by 5,5'-dithiobis(2-nitrobenzoic acid) and the concentration was calculated using a molar extinction coefficient of $1.36 \times 10^4\ M^{-1} \cdot cm^{-1}$ (16).

In all assays, we measured three or more samples at the same point. Using Student's *t*-test, p values of less than 0.01 were considered significant.

RESULTS

The rates of conjugated diene formation induced by Fe(II)-citrate in the presence of 0.5 mM DHLA homologues or the methyl ester derivative are shown in Fig. 2. The results were different for the lipid-dispersed and liposome systems. In the lipid-dispersed system, tetranorDHLA significantly inhibited the formation of conjugated dienes, whereas in the liposome system none of the homologues inhibited diene formation and the methyl ester derivative of DHLA enhanced their formation. In the absence of linoleic acid, the absorbance at 234 nm increased slightly due to Fe(III)-citrate formation, but the increase was negligible (less than 10% of the control).

Citrate produced opposite results in the lipid-dispersed and the liposome system. Citrate enhanced the formation of conjugated diene in the dispersed system, but conversely it inhibited conjugated diene formation in the liposome system. When desferal (0.5 mM) was added to the reaction mixture instead of citrate, it completely inhibited conjugated diene formation in both systems. These results indicate that all the conjugated dienes observed under our experimental

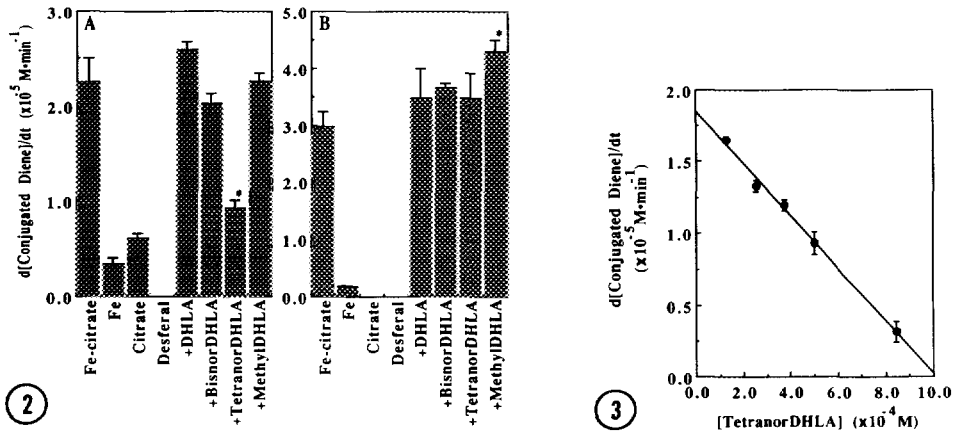


Fig. 2. The rate of conjugated diene formation induced by Fe(II)-citrate in the presence of DHLA homologues and derivative. **A**: lipid-dispersed and **B**: liposome system. The reaction was done at 25°C in a reaction mixture of 0.5 mM linoleic acid, 0.2 mM $FeSO_4$ and 0.4 mM citrate with or without 0.5 mM of the homologues or derivative. **Fe-citrate**: in the absence of DHLA homologues or derivative; **Fe**: $FeSO_4$ was added without citrate; **Citrate**: $FeSO_4$ was omitted; **Desferal**: desferal was added in place of citrate without adding iron. Values represent mean ± SD. *Significantly different from Fe(II)-citrate ($p<0.01$).

Fig. 3. The inhibition of conjugated diene formation in linoleic acid by tetranorDHLA in a lipid-dispersed system. The concentration of tetranorDHLA was changed under the same condition as in Fig. 2. TetranorDHLA inhibited formation of conjugated dienes depending on the concentration in a linear manner. Values represent mean ± SD.

conditions were catalyzed by iron, and both citrate and desferal could chelate the catalytic iron and remove it from the liposome system. In the aqueous phase citrate was unable to render iron non-catalytic.

We studied the concentration dependence of the inhibition of conjugated diene formation by tetranorDHLA (Fig. 3). TetranorDHLA linearly inhibited formation of conjugated dienes in a narrow concentration range. Higher concentrations of tetranorDHLA could not be used because the homologue had an absorbance at 280 nm that disturbed the measurement of conjugated dienes at higher concentrations.

To help elucidate the mechanism of the inhibition by tetranorDHLA, rates of oxygen consumption and Fe(II)-citrate oxidation were measured in the lipid-dispersed system (Fig.4). The rate of oxygen consumption tended to increase in the presence of DHLA homologues. In particular, tetranorDHLA increased the oxygen consumption rate three or four times compared with the Fe-citrate control (Fig. 4A). However the oxidation rate of Fe(II)-citrate was slower in the presence of tetranorDHLA than in other groups (Fig. 4B). These data indicate that tetranorDHLA was more rapidly oxidized than other homologues in the lipid-dispersed system.

DISCUSSION

To study the effect of DHLA homologues and a methyl ester derivative on iron-induced lipid peroxidation, we used commercial linoleic acid, without removal of preexisting lipid peroxide, and observed lipid peroxidation, which is mainly catalyzed by ferrous iron (7, 8). Fe(II)-

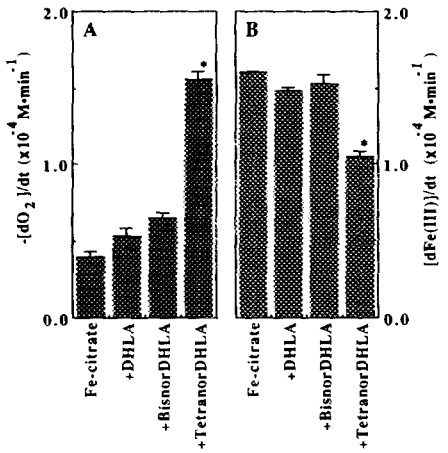


Fig. 4. Oxygen consumption and rate of oxidation of Fe(II)-citrate in the presence of various DHLA homologues. **A**: Oxygen consumption. **B**: Oxidation rate of Fe(II) to Fe(III)-citrate. Conditions as in Fig. 2. The shorter the chain was, the faster the oxygen consumption rate and the slower the oxidation rate of ferrous ion. Values represent mean ± SD. *Significantly different from other groups ($p<0.01$).

citrate was used as an initiator of lipid peroxidation because DHLA homologues and its derivative could reduce iron and make the reaction too complicated to analyze the mechanism. Therefore in the present study, we observed effects of the homologues on ferrous iron-catalyzed lipid peroxide-dependent lipid peroxidation (17-19).

Our results suggest that tetranorDHLA protected against the lipid peroxidation by the oxidation of tetranorDHLA (Fig. 2 and 4). We could not directly monitor the oxidation rate of DHLA homologues by measuring the absorbance at 330 nm (20) because Fe(III)-citrate strongly absorbs at around the same wavelength. Since Fe(III)-citrate is not able to oxidize DHLA homologues, and their oxidized forms are not able to oxidize ferrous iron within 1 min (data not shown), all oxygen is probably used to oxidize linoleic acid, Fe(II)-citrate and DHLA homologues. This hypothesis is supported by the fact that the rate of oxygen consumption (2×10^{-4} N•min^{-1}) was equal to the rate of lipid oxidation (0.5×10^{-4} N•min^{-1}) plus the rate of oxidation of ferrous iron (1.5×10^{-4} N•min^{-1}) in the Fe-citrate control. Therefore we can assume a simple model in our reaction system, that is, the electron acceptor is oxygen:

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

and in addition to lipid and ferrous iron the electron donors are DHLA homologues.

$$LH + O_2 \longrightarrow LOOH$$
$$Fe(II) \longrightarrow Fe(III) + e^-$$

For DHLA and bisnorDHLA oxygen consumption was approximately equal to the rate of lipid oxidation plus the rate of oxidation of ferrous iron, which indicated that they were not oxidized during the reaction. However tetranorDHLA was oxidized at the rate of about 1×10^{-4} M•min^{-1}. Therefore we inferred that tetranorDHLA was rapidly oxidized in the aqueous phase and inhibited

the formation of conjugated dienes by acting as a competitor for oxygen. It may be asked why tetranorDHLA does not inhibit lipid peroxidation in the liposome system. In Fe(II)-catalyzed lipid peroxidation it is critical that ionic iron is in the catalytic site (17, 21). In the lipid-dispersed system, tetranorDHLA could have free access to the lipid, but in the liposome system it might be difficult for tetranorDHLA to permeate into the liposome because of its hydrophilicity.

The reason for the increase in lipid peroxidation in the liposome system in the presence of the methyl ester derivative is not clear; it is possible that the methyl ester, being the most hydrophobic, incorporates into liposomes and reduces iron on the surface of the liposome; however, we did not test this mechanism.

The protection of DNA strand breaks by α-lipoic acid and DHLA against singlet oxygen has been suggested to be associated with metal chelation (22). DHLA homologues have two possible binding sites for iron, either the dithiol or the carboxyl group. Bonomi and Pagami (23) reported that DHLA and the reduced form of lipoamide can chelate ferric and ferrous ions. At pH 7.4, a thiolato complex is dominant for ferric ion and the carboxylato complex is dominant for ferrous ion (24). We could not observe the absorbance of the thiolato complex at 662 nm by absorption spectroscopy during the reaction. As an initiation species of lipid peroxidation, we used Fe(II)-citrate, which is also a carboxylato complex. Even if ferrous iron could exchange between citrate and DHLA homologues, the catalytic character of iron might not be changed because of the same carboxylato complex formation. But free dithiol could be at the site of oxidation of lipid, which may be beneficial for the protection of lipid peroxidation. However, this hypothesis does not explain the ineffectiveness of tetranorDHLA in the liposome system.

In the present study the effect of dithiols with different hydrophilicity on ferrous iron-induced lipid peroxidation was examined. TetranorDHLA, the most hydrophilic homologue, inhibited lipid peroxidation in a lipid-dispersed system by its own oxidation but was ineffective in a liposome system, and longer-chain homologues were unable to act as an antioxidant in either system. This suggests that in order to develop a novel antioxidant the antioxidant must have not only antioxidant capacity, but also site-specificity for the susceptible substance.

ACKNOWLEDGMENTS

Supported by National Institutes of Health (CA 47597) and ASTA Medica (Germany).

REFERENCES

1. Sies, H. (1985) Oxidative stress, Academic Press, London.

2. Aust, S. D., Chignell, C. F., Bray, T. M., Kalyanaraman, B., and Mason, R. P. (1993) *Toxicol. Appl. Pharmacol.* **120**, 168-178.

3. Halliwell, B. , and Gutteridge, J. M. C. (1989) Free radicals in biology and medicine, Clarendon press, Oxford.

4. Stadtman, E. R. (1992) *Science* **257**, 1220-1224.

5. Halliwell, B. , and Gutteridge, J. M. C. (1990) *in* Methods in Enzymology: Role of free radicals and catalytic metal ions in human disease: an overview (Packer, L., and Glazer, A. N., Eds.), Vol. 186, pp. 1-85. Academic Press, San Diego.

6. Millar, D. M., Buettner, G. R., and Aust, S. D. (1990) *Free Rad. Biol. Med.* **8**, 95-108.

7. Schaich, K. M. (1992) *Lipids* **27**, 209-218.

8. Minotti, G., and Aust, S. D. (1992) *Lipids* **27**, 219-226.

9. Stadtman, E. R., and Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005-2008.

10. Suzuki, Y. J., Tsuchiya, M., and Packer, L. (1991) *Free Rad. Res. Comms.* **15**, 225-263.

11. Suzuki, Y. J., Tsuchiya, M., and Packer, L. (1993) *Free Rad. Res. Comms.* **18**, 115-122.

12. Kawabata, T., Ogino, T., and Awai, M. (1989) *Biochim. Biophys. Acta.* **1004**, 89-94.

13. Gutteridge, J. M. C., Rowley, D. A., and Halliwell, B. (1981) *Biochem. J.* **199**, 263-265.

14. Grootveld, M., Bell, J. D., Halliwell, B., Aruoma, O. I., Bomford, A., and Sadler, P. J. (1989) *J. Biol. Chem.* **264**, 4417-4422.

15. Pryor, W. A., and Castle, L. (1984) *in* Methods in Enzymology: Chemical methods for the detection of lipid hydroperoxides (Packer, L., Eds.), Vol. 105, pp. 293-299. Academic Press, San Francisco.

16. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77.

17. Fujii, T., Hiramoto, Y., Terao, J., and Fukuzawa, K. (1991) *Arch. Biochem. Biophys.* **284**, 120-126.

18. Fukuzawa, K., Fujii, T., and Mukai, K. (1991) *Arch. Biochem. Biophys.* **290**, 489-496.

19. Fukuzawa, K., and Fujii, T. (1992) *Lipids* **27**, 227-233.

20. Furr, H. C., Smith, J. C. H., Harrison, E. H., Chang, H.-H., Spence, J. T., Wright, L. D., and McCormick, D. B. (1979) *in* Methods in Enzymology: Chromatographic and spectral properties of lipoic acid and its metabolites (McCormick, D. B., and Wright, L. D., Eds.), Vol. 62, Vitamins and Coenzymes Part D, pp. 129-135. Academic Press, New York.

21. Schaich, K. M., and Borg, D. C. (1988) *Lipids* **23**, 570-579.

22. Devasagayam, T. P. A., Subramanian, M., Pradhan, D., and Sies, H. (1993) *Chem.-Biol. Interactions.* **86**, 79-92.

23. Bonomi, F., and Pagani, S. (1986) *Eur. J. Biochem.* **155**, 295-300.

24. Bonomi, F., Pagani, S., Cariati, F., Pozzi, A., Guido, C., Cristiani, F., Nurchi, V., Russo, U., and Zanoni, R. (1992) *Inorg. Chim. Acta.* **195**, 109-115.