Temporal Relationship of Free Radical-induced Lipid Peroxidation and Loss of Latent Enzyme Activity in Highly Enriched Hepatic Lysosomes*

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(Received for publication, May 23, 1983)

Lipid peroxidation has been suggested to be associated with a variety of pathological processes such as liver necrosis, hemolytic anemia, lung damage (1-4), and, more recently, ischemic heart disease (5, 6). The peroxidation of lipids may involve the direct reaction of oxygen radicals with polyunsaturated fatty acids resulting in deterioration of the lipid (4). Membrane lipids are major sites of lipid peroxidation damage due to the presence of polyunsaturated fatty acids in their membrane phospholipids. The pathological consequence of lipid peroxidation may thus reflect the alterations of membrane integrity and/or membrane-associated function in subcellular organelles such as mitochondria, microsomes, and lysosomes.

Lysosomes contain many hydrolytic enzymes and their liberation may result in extensive intracellular digestion. Work of Allison and Young (7) provided evidence that hydrolases released in vivo in cells can cause severe damage to cellular structure. Thus, the susceptibility of the lysosomal membrane to lipid peroxidation is of apparent importance to cellular pathology. Studies of the effect of lipid peroxidation induced by irradiation (8) and by oxidation of NADPH by the microsomal monooxygenase system (9) on lysosomal membranes have been reported. However, in those studies of modestly enriched lysosomal preparations, there was heavy contamination by other subcellular organelles. In an attempt to circumvent such cross-contamination, we have used highly purified "native" hepatic lysosomes prepared by free flow electrophoresis to study their susceptibility to free radical-induced lipid peroxidation. The free radicals were generated during the autoxidation of DHP in the presence of Fe^{3+}-ADP.

MATERIALS AND METHODS

Chemicals—DHF, 2-thiobarbituric acid, FeCl_3-6H_2O, p-nitro-phenyl-N-acetyl-α-D-glucosaminide, glucose-6-phosphate, cytochrome c (Type VI), mannitol, triethanolamine, and αToc were purchased from Sigma. 2,5-Dimethylfuran and 1,4-diazabicyclo[2.2.2]octane were obtained from Aldrich. Superoxide dismutase (specific activity 3,000 units/mg) was isolated from bovine erythrocytes and assayed as described by McCord and Fridovich (10). Catalase (specific activity 40,000 units/mg) was obtained from Worthington and was treated to remove contaminating superoxide dismutase and preservatives as previously described (11).

Preparation and Characterization of Lysosomes—Lysosomes were isolated by a free flow electrophoresis procedure similar to that described by Henning and Heidrich (12) and Beckman et al. (13). Sprague-Dawley rats (200-300 g) were decapitated and the livers were perfused with ice-cold 0.25 M sucrose, 0.003 M MgCl_2, 0.001 M EDTA, 0.01 M MOPS, pH 7.2. All subsequent steps were performed at 0-4°C unless stated otherwise. The perfused livers were homogenized in the above buffer first with a Virtis tissue disrupter for 2 x 3 s (at setting 50) followed by two up and down strokes in a Thomas Teflon pestle homogenizer. After centrifugation for 15 min, the 5-20,000 × g pellet was resuspended and extracted in 0.12 M K-glucanate, 0.05 M sucrose, 0.001 M EDTA, 0.01 M MOPS (pH 7.4) and centrifuged again at 20,000 × g for 15 min. The replacement of KCl by K-glucanate in this step of extraction resulted in more latent lysosomes than those obtained by the method of Beckman et al. (13). The pellet was then "washed" twice with 0.25 M sucrose, 0.001 M EDTA, 0.01 M MOPS (pH 7.4). The final pellet was resuspended in the electrophoresis buffer (0.3 M sucrose, 0.01 M triethanolamine, 0.001 M EDTA, and 0.01 M acetate adjusted to pH 7.4 by NaOH) and aggregated particles were removed by centrifugation at 1,000 × g for 10 min. The supernatant was then applied to a free flow electrophoresis unit (Desaga 48, Heidelberg, West Germany). The electrode buffer consisted of 0.1 M triethanolamine and 0.1 M acetate, pH 7.4 (NaOH). The unit was run at 800 V and 100 mA at 3°C. The sample pump was set at 4 ml/min and the collecting pump was at a setting of 3.0. After electrophoresis, the fractions were concentrated by immediate centrifugation at 20,000 × g for 20 min and analyzed for free and total specific β-N-acetylgalactosaminidase activities as described by

* This work was supported by National Heart, Lung, and Blood Institute Grants T32-HL-07244 and HL-28885. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Free Radical-induced Lysosomal Lipid Peroxidation

Ruth and Weglicki (14). Percentage free activity was calculated as activity in 0.25 M sucrose/activity in 0.1% Triton X-100 $\times$ 100. Fractions were isolated between tubes 8 to 40. The elution profile of the $\beta$-N-acetylglucosaminidase activities was similar to that reported earlier (13), and fractions highly enriched in $\beta$-N-acetylglucosaminidase were nearer to the anode. Marker enzymes in the various fractions were assayed as an indicator of the degree of subcellular particulate separation. Glucose-6-phosphatase was assayed according to the method of Baginski et al. (15). Cytochrome oxidase was assayed as an indicator of the degree of subcellular purity of the fractions. The purified lysosomes were resuspended in the reaction buffer (0.12 M KCl, 0.05 M sucrose, 0.01 M potassium phosphate, pH 7.2) and were used immediately for free radical-induced lipid peroxidation studies. The incubation mixture usually contained 150-200 $\mu$g of lysosomal protein, 0.1 mM FeCl$_3$, 1 mM ADP, and 3.33 mM DHF in 1 ml of the reaction buffer. Specific conditions or additional components in the incubation mixture are described in the text. Reactions were initiated by the final additions of Fe$^{3+}$-ADP and DAF. Incubations were carried out at 37 °C in air in a shaking water bath. At the indicated times of incubation, aliquots of the reaction mixtures were assayed for free and total activities of $\beta$-N-acetylglucosaminidase.

The initial 5 to 7 fractions containing highly enriched lysosomes ($\beta$-N-acetylglucosaminidase $>30$-fold, cytochrome oxidase $<0.3$-fold) were pooled and pelleted at 20,000 $\times$ g for 20 min. The purified lysosomes were resuspended in the reaction buffer and were used immediately for free radical-induced lipid peroxidation studies. The incubation mixture usually contained 150-200 $\mu$g of lysosomal protein, 0.1 mM FeCl$_3$, 1 mM ADP, and 3.33 mM DHF in 1 ml of the reaction buffer. Specific conditions or additional components in the incubation mixture are described in the text. Reactions were initiated by the final additions of Fe$^{3+}$-ADP and DAF. Incubations were carried out at 37 °C in air in a shaking water bath. At the indicated times of incubation, aliquots of the reaction mixtures were assayed for free and total activities of $\beta$-N-acetylglucosaminidase.

TABLE I

| Steps          | $\beta$-N-acetylglucosaminidase | Cytochrome oxidase | Glucose-6-phosphatase |
|----------------|--------------------------------|-------------------|-----------------------|
| Specific activity | Fold enrichment | Specific activity | Fold enrichment | Specific activity | Fold enrichment |
| Total homogenate | 18.5 ± 1.9 | 1 | 278 ± 28 | 1 | 66.5 ± 5.6 | 1 |
| 5–20,000 $\times$ g pellet | 169.0 ± 19.8 | 9.1 | 407 ± 46 | 1.47 | 70.3 ± 8.1 | 1.06 |
| Purified fractions by free flow electrophoresis | 1014 ± 126 | 54.8 | 67 ± 11 | 0.24 | 17.6 ± 3.1 | 0.26 |

*a Specific activity = nmol/min of protein.

RESULTS AND DISCUSSION

Characterization of the Purified Lysosomes—Our methods of tissue extraction and differential centrifugation routinely produced enriched (9- to 10-fold) lysosomes which were highly latent (16.7 ± 3.2% free). By using free flow electrophoresis (Table I), we were able to isolate more highly purified (up to 60-fold enriched) native rat liver lysosomes with very low percentage free activities (about 20% free) of $\beta$-N-acetylglucosaminidase. The purified lysosomes were observed to exhibit relatively low levels of cytochrome oxidase and glucose-6-phosphatase activities (Table I). These fractions contained only trace levels of catalase activity (data not shown). Thus, in agreement with the reports of other groups (12, 19), lysosomes isolated by free flow electrophoresis were reasonably free of other subcellular contamination.

Peroxidation of Lysosomes—Aerobic oxidation of DHF generates large steady state levels of superoxide anions (O$_2^-$), which have been suggested to generate additional active oxygen radicals capable of inducing lipid peroxidation (20, 21). Fig. 1a shows that incubation of the lysosomes with the buffer alone for 30 min produced very low levels of lipid peroxide (1.66 ± 0.64 nmol/mg of protein). Additions of Fe$^{3+}$-ADP alone or DHF alone only generated modest increases in peroxidation products. However, when the lysosomes were incubated with DHF in the presence of Fe$^{3+}$-ADP, lipid peroxidation, as indicated by MDA formation (thiobarbituric acid-reacting products), was greatly and significantly stimulated (63.7 ± 6.6 nmol/mg of protein, p < 0.001), suggesting that Fe$^{3+}$-ADP might function as a pro-oxidant in this system (22). The effect of the induced peroxidation on lysosomal membrane integrity was examined (Fig. 1b). Incubation of the lysosomes in buffer alone resulted in 29.3 ± 2.8% free activity, indicating that the lysosomes still remained relatively intact. Neither Fe$^{3+}$-ADP alone nor DHF alone significantly in-
creased the percentage free activities. However, addition of DHF and Fe$^{3+}$-ADP together resulted in greatly elevated percentage free activity of $\beta$-N-acetylglucosaminidase (81.1 ± 5.6% free) that was highly significant ($p < 0.001$). The possibility of activation of $\beta$-N-acetylglucosaminidase by the radical-generating system was ruled out as follows. At the end of the incubation, samples incubated with or without the radical-generating system exhibited similar total activities of $\beta$-N-acetylglucosaminidase which did not change appreciably over the time of incubation.

Time-course studies were conducted to compare the rate of lipid peroxidation with changes in free activity of $\beta$-N-acetylglucosaminidase (Fig. 2). With the additions of both Fe$^{3+}$-ADP and DHF, lipid peroxidation occurred very rapidly and appeared to have reached a maximum after 10 min of incubation. However, the loss of lysosomal latency occurred at a relatively slower rate and reached a maximum at about 30 min. As a comparison, only an insignificant increase occurred in the level of MDA formed (from 1.13 ± 0.16 to 1.66 ± 0.64 nmol of MDA/mg of protein) and a modest, though significant ($p < 0.05$), increase (from 24.2 ± 2.0 to 29.3 ± 2.8%) in percentage free activity of $\beta$-N-acetylglucosaminidase was observed in the control samples over the same period of incubation.

Loss of lysosomal latency is known to be pH dependent (23, 24). In a separate experiment, incubation of the purified lysosomes for 30 min at 37 °C in a buffer of 0.12 mM KCl, 0.05 mM sucrose, and 0.01 mM potassium phosphate, pH 5, resulted in 60–70% free activity of $\beta$-N-acetylglucosaminidase. The loss of lysosomal integrity under the acidic condition is probably due to endogenous lipolytic degradation of the lysosomal membrane (13). In neutral pH, a similar incubation procedure resulted in only a modest increase in percentage free activity (Fig. 2). Presumably, lysosomal lipolytic enzymes are active in acidic pH range (pH 4 to 5), but not in neutral pH range (25). However, in neutral pH, when the lysosomes were incubated with DHF plus Fe$^{3+}$-ADP, lipid peroxidation was induced and the loss of latency followed (Figs. 1 and 2). If any of the components were absent, lipid peroxidation did not occur, nor did the subsequent loss of the latency (Fig. 1). If all the components were present and α-tocopherol was introduced to neutralize the free radicals (Table III), lipid peroxidation was inhibited and the loss of latency was also greatly reduced. Thus, these data strongly suggest that the loss of lysosomal latency is more of a consequence of the free radical-induced lipid peroxidation and not due to the nonspecific combined effect of the components.

**Lack of Protection by Various Active Oxygen Scavengers against Lipid Peroxidation**—Once formed, O$_2^-$ leads to the generation of other active oxygen species such as hydroxyl radical (·OH), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$) through the following reaction scheme (9, 26, 27).

\[
\begin{align*}
\text{O}_2^- + \text{O}_2^+ + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_3 \\
\text{H}_2\text{O}_2 + \text{O}_2^- & \rightarrow \text{O}_3^- + \cdot\text{OH} + \text{OH}^- \\
\text{H}_2\text{O}_2 + \text{O}_2^- & \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_3
\end{align*}
\]

The Reaction b may be catalyzed by Fe$^{3+}$-ADP as follows (9, 27).

\[
\text{O}_2^- + \text{Fe}^{3+}\text{-ADP} \rightarrow \text{O}_3^- + \text{Fe}^{2+}\text{-ADP}
\]

\[
\text{Fe}^{2+}\text{-ADP} + \text{O}_3^- \rightarrow \text{Fe}^{3+}\text{-ADP} + \text{OH}^- + \text{OH}
\]

In an attempt to identify the radical species directly responsible for the induced peroxidation, scavengers for different active oxygen radicals were added to the lysosomal samples before the addition of the radical-generating system. As indicated in Table II, scavengers for O$_2^-$, H$_2$O$_2$, ·OH, and ¹O$_2$ did not appear to prevent the lipid peroxidation or the loss of latency activity of $\beta$-N-acetylglucosaminidase. Longer preincubation (up to 5 min at 37 °C) of the scavengers with the lysosomes did not affect the subsequent results presented in Table II. None of the scavengers tested altered the specific activity of $\beta$-N-acetylglucosaminidase.

In studying the effect of lipid peroxidation initiated by the oxidation of NADPH by liver microsomes on lysosomes, Fong et al. (9) presented evidence that ·OH was the radical species responsible for oxidative attack of the membrane lipids. To the contrary, our present data indicate that mannitol and ethanol, both ·OH scavengers, were without effect. One possible interpretation of our results is the inaccessibility of the site of ·OH generation to the scavengers. The O$_2^-$, H$_2$O$_2$, and Fe$^{2+}$/Fe$^{3+}$ that are produced may enter the lysosomal membrane freely and generate ·OH there, but the scavengers may not enter as efficiently and thus no protection would be provided. We include 0.05 mM sucrose in our system, and since sucrose was reported to scavenge ·OH at a tremendous rate ($K = 2.8 \times 10^9$ M$^{-1}$ s$^{-1}$) at low pH (28), we investigated the effect of ·OH scavengers in a system containing phosphate buffered saline, pH 7.4, without sucrose. In this system, 6 mM mannitol and 0.85 mM ethanol were also without protective effect against lysosomal lipid peroxidation. Alternatively, the radical species promoting the lipid peroxidation might be a
Tocopherol is well known as an antioxidant; however, the extent of its protective effect is less clear. The mechanism of the anti-oxidative action of tocopherol is not known in detail (26, 30); α-tocopherol can act as a free radical scavenger and also as a singlet oxygen quenching agent (26). Singlet oxygen has been postulated to promote lipid peroxidation (26, 32). Since 1,4-diazabicyclo[2,2,2]octane and 2,5-dimethylfuran, both being singlet scavengers, were with-
out major effect in our system, the inhibitory effect of α-tocopherol might be due to its ability to interrupt the propagation of the free radical chain reaction already initiated in the membrane lipids (30). Our observations seem to agree with the proposal of Lucy (33) which suggests that tocopherol physicochemically forms a complex with the fatty acyl chains of polyunsaturated phospholipids, particularly those derived from arachidonic acid, and probably at least in polyunsaturated fatty acids with the following functional consequences: i) inhibition of peroxidative destruction of polyunsaturated fatty acids in the membranes; ii) prevention of permeability (or leakage) of biological membranes containing relatively high levels of polyunsaturated fatty acids; and iii) possible prevention of degradation of the membrane phospholipids by membrane-bound phospholipase.

Our experiments (Fig. 2) indicate that the formation of lipid peroxides in the membrane preceded the disintegration of the lysosomes. The data, however, do not distinguish whether the accumulation of the lipid peroxides (or the loss of membrane polyunsaturated fatty acids) and/or a subsequent enzymatic process, such as lipolysis, would be directly responsible for the degradation of the lysosomal membrane.

In conclusion, our results clearly demonstrate the susceptibility of lysosomes to lipid peroxidation induced by free radicals. Since free radicals are believed to exist in vivo (34) and are capable of promoting lipid peroxidation via a NAPD-dependent microsomal enzyme system in vitro (9, 35), we have carried out a preliminary study of the susceptibility of the purified lysosomes to free radicals generated by the microsomal NADPH oxidase system. The liver microsomes were prepared as previously described (9, 35). In a typical experiment, purified lysosomes (0.2 mg of protein) in 1 ml of the same buffer described in Fig. 1 were incubated with 0.1 mg of the microsomes in the presence of 0.4 mM NAPD and Fe²⁺-ADP (0.1 mM FeCl₂, 1 mM ADP). Lipid peroxidation was greatly induced as indicated by the formation of 16–20 nmol of MDA in 30 min at 37 °C. Concomitantly, the lysosomal latency was also lost as indicated by the elevated percentage free (80–90%) activity of β-N-acetylgalactosaminidase. The lysosomes incubated with the microsomes alone or in systems containing no NAPD or Fe²⁺-ADP exhibited no more than 5% of the MDA formed in the complete system and showed only a modest level of free activity of β-N-acetylgalactosaminidase (33–40% free). These data further support the conclusion that the observed susceptibility of the purified lysosomes to the free radicals generated by the DHF plus Fe³⁺-ADP system is not unique to that system; other systems, whether enzymatic or nonenzymatic, capable of generating free radicals may induce similar peroxidative damage on the lysosomal membrane. Our data demonstrate a similar facilitary effect of Fe²⁺-ADP on the peroxidative reactions as reported by other studies (9, 35). The concentration of inorganic iron in the liver cytosol has been estimated in the range of 1.0×10⁻⁶ to 1.28×10⁻⁴ M, whereas the cytosolic concentration of ADP is believed to be in the millimolar range (36). Since the concentrations of both components are at levels compatible with promoting the process described, it is reasonable to consider the possibility that a similar destructive reaction on lysosomes may be catalyzed in vivo by certain free radical-generating oxidoreductase systems, especially when free radical scavengers such as α-tocopherol are absent.

Acknowledgment—We wish to thank Dr. Paul B. McCay for his helpful discussions and review of this work.

REFERENCES

1. Plaa, G. L., and Witschi, H. (1976) Annu. Rev. Pharmacol. Toxicol. 16, 125–141
2. Fridovich, I. (1979) in Oxygen Free Radicals and Tissue Damage pp. 1–42, Elsevier/North-Holland, New York
3. McCay, P. B. (1980) Fed. Proc. 40, 173
4. Tappel, A. L. (1975) Fed. Proc. 34, 1870–1874
5. Guarnieri, G., Flamigni, F., and Calderara, C. M. (1980) J. Mol. Cell. Cardiol. 12, 797–808
6. Hess, M. L., Manson, N. H., and Okabe, E. (1982) Can. J. Physiol. Pharmacol. 60, 1382–1386
7. Allison, A. C., and Young, M. R. (1969) in Lysosomes in Biology and Pathology (Dingle, J. T., and Fell, H. B., eds) Vol. 2, pp. 600–628, American Elsevier Publishing Co., New York
8. Wills, E. D., and Wilkinson, A. E. (1966) Biochem. J. 99, 657–666
9. Fong, K.-L., McCay, P. B., Poyer, J. L., Keele, B. B., and Misra, H. (1973) J. Biol. Chem. 248, 7792–7797
10. McCord, J. M., and Fridovich, I. (1969) J. Biochem. (Tokyo) 244, 6049–6055
11. Misra, H. P. (1974) J. Biochem. (Tokyo) 249, 2151–2155
12. Henning, R., and Heidrich, H.-G. (1974) Biochim. Biophys. Acta 345, 326–335
13. Beckman, J. K., Owens, K., and Weglicki, W. B. (1981) Lipo. 16, 796–799
14. Ruth, R. C., and Weglicki, W. B. (1978) Biochem. J. 172, 163–172
15. Baginski, E. S., Foa, P. P., and Zark, B. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) Vol. 2, pp. 876–886, Academich Press Inc., New York
16. Wharton, F. C., and Tzagoloff, A. (1967) Methods Enzymol. 10, 245–250
17. Lowry, M. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–270
18. Wills, E. D. (1969) Biochem. J. 113, 315–324
19. Stahn, R., Maier, K. P., and Hannig, K. (1970) J. Cell Biol. 46, 576–591
20. Halliwell, B. (1977) Biochem. J. 163, 441–448
21. Goldberg, B., and Stern, A. (1977) Arch. Biochem. Biophys. 178, 218–225
22. Chicarotto, E., Olivero, J., Albemo, E., Poli, G., Gravels, E., and Dangani, M. U. (1981) Experientia (Basel) 37, 396–397
23. Burton, R., and Lloyd, J. B. (1978) Biochim. Biophys. Acta 160, 631–638
24. Wattiaux, R. (1977) in Mammalian Cell Membrane (Jameson, G., and Robinson, D. M., eds) Vol. 2, pp. 165–184, Butterworths, Boston
25. Tappel, A. L. (1969) in Lysosomes in Biology and Pathology (Dingle, J. T., and Fell, H. B., eds) Vol. 2, pp. 207–227, American Elsevier Publishing Co., New York
26. McCay, P. B., Fong, K.-L., Lai, E. K., and, King, M. M. (1978) in Tocopherol, Oxygen and Biomenoners (deDuve, C., and Hayashi, O., eds) pp. 41-57, Elsevier/North-Holland Biomedical Press, Amsterdam.
27. Singh, A. (1982) Can. J. Physiol. Pharmacol. 60, 1330–1345
28. Scholes, G., and Willson, R. L. (1967) Faraday Soc. Trans. 63, 2983–2993
29. Svingen, B. A., Buge, J. A., O'Neal, F. O., and Aust, S. D. (1979) J. Biol. Chem. 254, 8592–8599
30. McCay, P. B., and King, M. M. (1980) in Vitamin E, A Comprehensive Treatise (Machlin, L. J., ed) pp. 286–317, Marcel Dekker, Inc., New York
31. Seeman, P. (1972) Pharmacol. Rev. 24, 584–655
32. Kellogg, E. W., III, and Fridovich, I. (1975) J. Biol. Chem. 250, 8812–8817
33. Lucy, A. J. (1972) Ann. N. Y. Acad. Sci. 203, 4–11
34. McCay, P. B., and Poyer, J. L. (1976) in The Enzymes of Biological Membranes (Martenos, A., ed) Vol 4, pp. 239–256, Plenum Publishing Corp., New York
35. May, H. E., and McCay, P. B. (1968) J. Biol. Chem. 243, 2288–2295
36. Fong, K.-L., McCay, P. B., and Poyer, J. L. (1976) Chem. Biol. Interact. 15, 77–88
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*J. Biol. Chem.* 1983, 258:13733-13737.

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