ENHANCEMENT OF NADPH-DEPENDENT LIPID
PEROXIDATION ACTIVITY BY ETHYLENE-
DIAMINETETRAACETIC ACID (EDTA) IN THE PRESENCE
OF FERROUS ION IN RABBIT LIVER MICROSONES

Mitsukazu KITADA, Tetsuya KAMATAKI and Haruo KITAGAWA
Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences,
Chiba University, Chiba 280, Japan
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Abstract—The addition of EDTA to the incubation mixture containing rabbit liver
microsomes and ferrous ion resulted in 2-fold increase of lipid peroxidation activity.
Such an enhancement was not observed in rat liver microsomes. The maximum
lipid peroxidation activity seen in rabbit microsomes in the presence of EDTA and
ferrous ion was about 80% that seen in rat liver microsomes. From these results, it
is likely that low lipid peroxidation activity in rabbit liver microsomes may account
for the insufficiency of an EDTA-like factor(s) in rabbit liver microsomes.

Unsaturated fatty acids in liver microsomes undergo peroxidative degradation. This
lipid peroxidation reaction is generally accepted as the cause of hepato-toxicity with ingestion
of certain chemical compounds.

Gram and Fouts (1) first reported that there were differences in lipid peroxidation in
the 9,000 x g supernatant fraction of liver homogenates from several animal species; high
lipid peroxidation activity in the rat and very low activity in the rabbit. In a previous paper
(2), we reported essentially the same species difference in the activity of lipid peroxidation in
microsomes.

With respect to the enzyme which concerns with NADPH-dependent lipid peroxidation
in liver microsomes, Pederson and Aust (3) and Pederson et al. (4) concluded that NADPH-
cytochrome c reductase is the enzyme which transfers electrons from NADPH to the site of
the reaction. They also demonstrated the possibility that there is an EDTA-like factor(s)
which activates lipid peroxidation in liver microsomes since EDTA and ferrous ion as well
as purified NADPH-cytochrome c reductase and microsomal lipids were absolute require-
ments for reconstitution of lipid peroxidation activity.

As we reported previously (2), there were no close correlations between lipid peroxidation
activity and NADPH-cytochrome c reductase activity in liver microsomes from various
animal species, indicating that the species difference in lipid peroxidation activity is due to
factors other than NADPH-cytochrome c reductase.

In this communication we would like to report on the requirement of EDTA for
maximum activity of lipid peroxidation in rabbit liver microsomes.

MATERIALS AND METHODS

New Zealand male rabbits weighing 1.75 to 1.90 kg and male Wistar rats weighing 234
to 293 g were maintained on commercial chow, Nippon Clea Co., Japan. The animals were deprived of food for approximately 18 hr prior to sacrifice but were provided water *ad libitum*. Liver microsomes of the animals were prepared by a method previously described (5). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I) were purchased from Boehringer Mannheim Co. Thiobarbituric acid was purchased from Daiichi Pure Chemicals Co., Japan and a 0.67% solution was prepared as described previously (6). Other reagents were purchased from commercial sources and were used without further purification. Unless otherwise stated, an incubation mixture consisted of microsomes (0.63 to 1.00 mg protein), Tris-HCl (100 mM, pH 7.4) and an NADPH-generating system in a final volume of 1.0 ml. NADPH-generating system contained NADP (0.33 mM), glucose 6-phosphate (8 mM), glucose 6-phosphate dehydrogenase (0.045 unit) and MgCl$_2$ (6 mM). When necessary, EDTA and/or ferrous ion (as Fe(NH$_4$)$_2$(SO$_4$)$_2$-6H$_2$O) were added in the incubation mixture. All incubations were carried out aerobically at 37°C for 30 min. The microsomal protein was determined according to the method of Lowry et al. (7), and bovine serum albumin was used as the standard. Lipid peroxidation activity was expressed in terms of thiobarbituric acid (TBA) value (absorbance at 532 nm due to malonaldehyde formation/mg of microsomal protein added in the incubation mixture). All values in the figures are means from duplicate determinations.

RESULTS

Pederson and Aust (3) and Pederson *et al.* (4) reported that NADPH-cytochrome *c* reductase is the sole enzyme transferring electrons from NADPH to the site of lipid peroxidation and that ferrous ion and EDTA were essential requirements for reconstituting NADPH-dependent lipid peroxidation activity. The requirement of EDTA in the reconstituted lipid peroxidation system suggested the existence of a factor(s) acting as an activator of lipid peroxidation, in a manner similar to EDTA in liver microsomes.

We observed that lipid peroxidation activity in rabbit liver microsomes is significantly lower than that in rat liver microsomes (2). Since there was not much difference in the NADPH-cytochrome *c* reductase activity between rats and rabbits (2), it seemed likely that a

![Fig. 1. Effects of EDTA on NADPH-dependent lipid peroxidation in microsomes from rats and rabbits. Liver microsomes from rats (■) and rabbits (○) were incubated in the presence of various concentrations of EDTA and in the presence (●, ■) or absence (○, ○) of added ferrous ion (20 μM). Other experimental conditions are described in Materials and Methods.](image-url)
factor(s) like EDTA was responsible for limiting lipid peroxidation in rabbit liver microsomes. The results indicating the requirement of EDTA for maximum activity of rabbit liver microsomal lipid peroxidation are shown in Fig. 1. When EDTA was added to the incubation mixture containing known amounts of ferrous ion, EDTA enhanced lipid peroxidation markedly although such only slightly enhanced lipid peroxidation activity without added ferrous ion. On the other hand, as shown in the same figure, EDTA exhibited only

**Fig. 2.** Effect of ferrous ion on the EDTA-induced change of lipid peroxidation activity in rabbit liver microsomes. Various concentrations of ferrous ion were added to the incubation mixture containing rabbit liver microsomes and 0 μM (●), 30 μM (○), 60 μM (△) or 100 μM (▲) EDTA. Other experimental conditions are described in Materials and Methods.

**Fig. 3.** Effects of ferrous ion and/or EDTA on rat liver microsomal lipid peroxidation. Experimental conditions are as in Fig. 2 except that rat liver microsomes were used instead of rabbit liver microsomes. For key to symbols, see Fig. 2.
inhibition of lipid peroxidation regardless of the presence of ferrous ion in rat liver microsomes.

The enhancement by EDTA of lipid peroxidation in rabbit liver microsomes was further supported by the experiment shown in Fig. 2. Here, various concentrations of ferrous ion were added in the incubation mixture containing fixed amounts of EDTA. In the absence of EDTA, addition of ferrous ion gradually increased lipid peroxidation activity with the concentrations added, and the activity at 150 μM ferrous ion was about 2.1-fold higher than that obtained without added ferrous ion. In the presence of 30 μM EDTA, lipid peroxidation was inhibited when ferrous ion was absent, however, with increase in the amounts of ferrous ion in the incubation mixture, lipid peroxidation activity increased to a greater extent. The highest lipid peroxidation activity in the presence of 30 μM EDTA was obtained at about 50 to 60 μM ferrous ion concentration and the maximum activity was about 5.3-fold higher than the corresponding control activity. Thus, the ratio of maximum level of lipid peroxidation activity in the presence of EDTA to that in the absence of EDTA was approximately 2:1. In another words, EDTA activated lipid peroxidation in rabbit liver microsomes even in the presence of excess ferrous ion. The result from a similar experiment using rat liver microsomes is shown in Fig. 3. It is evident that the addition of EDTA exhibits only inhibition of lipid peroxidation and the inhibition by EDTA is reversed by the addition of ferrous ion. The maximum levels of lipid peroxidation in the presence of various concentrations of EDTA and 150 μM concentration of ferrous ion were much the same.

In order to determine whether or not the enhancement of lipid peroxidation by EDTA is a reversible phenomenon, rabbit liver microsomes were prepared from livers which had been homogenized with (——) or without (—) 10 mM EDTA. The microsomes thus obtained were washed once with 1.15% KCl, and used for the assay of lipid peroxidation. The incubations were carried out in the presence of various concentrations of ferrous ion and in the presence (●) or absence (○) of extra added 30 μM EDTA. Other experimental conditions are described in Materials and Methods.
can be seen in Fig. 4, addition of EDTA enhanced lipid peroxidation even in microsomes which had been previously treated with EDTA. Therefore, it can be concluded that EDTA enhances lipid peroxidation directly and reversibly.

DISCUSSION

As was shown in Figs. 1 and 2, addition of excess amounts of EDTA inhibited lipid peroxidation, suggesting that the presence of free ferrous ion as well as EDTA-ferrous ion complex is required for maximal lipid peroxidation activity in rabbit liver microsomes.

It was of interest to determine whether or not other chelating agents such as 2,2'-bipyridine and 1,10-phenanthroline are capable of exhibiting the effect similar to EDTA. However, our preliminary studies indicated that EDTA cannot be substituted with these chelating agents (data not shown).

It was recently demonstrated that there is another factor(s) which inhibits lipid peroxidation in microsomes from several animal species (8, 9). Our recently acquired data using a reconstituted lipid peroxidation system which contains purified NADPH-cytochrome c reductase from detergent-solubilized rabbit liver microsomes showed that EDTA and ferrous ion were absolutely required for lipid peroxidation activity, and that the lipid peroxidation activity of the reconstituted system was 100 to 150 times greater than that of microsomes when the activities were expressed on the unit of NADPH-cytochrome c reductase basis. These results suggest that in rabbit liver microsomes, most of the lipid peroxidation capacity is inhibited by the lipid peroxidation inhibitor. Therefore, it is highly likely that apparent lipid peroxidation activity seen on rabbit liver microsomes is controlled by two factors; one an EDTA-like activator and the other a lipid peroxidation inhibitor.

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REFERENCES

1) Gram, T.E. and Fouts, J.R.: Arche Biochem. Biophys. 114, 331 (1966)
2) Kamataki, T. and Kitagawa, H.: Biochem. Pharmacol. 23, 1915 (1974)
3) Pederson, T.C. and Aust, S.D.: Biochem. biophys. Res. Commun. 48, 789 (1972)
4) Pederson, T.C., Buige, J.A., and Aust, S.D.: J. Biol. Chem. 248, 7134 (1973)
5) Kamataki, T. and Kitagawa, H.: Japan. J. Pharmacol. 24, 195 (1974)
6) Kamataki, T. and Kitagawa, H.: Biochem. Pharmacol. 22, 3199 (1973)
7) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: J. Biol. Chem. 193, 265 (1951)
8) Takeshige, K. and Minakami, S.: J. Biochem., Tokyo 76, 1151 (1974)
9) Talcott, R.E., Denk, H., Eckerstorfer, R. and Schenkman, J.B.: Chem. Biol. Interns. 12, 355 (1976)