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Effects of EDTA on Stability of Drug Metabolizing Enzymes in Liver Microsomes of Rats

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Effects of ethylenediamine tetraacetic acid (EDTA) on aging-induced changes in activities of drug metabolizing enzymes were investigated. Aging of microsomes at 45° produced a reduction of lipid peroxidation while aging of 9000 $\times g$ supernatant fraction at 45° resulted in a marked increase in the formation of lipid peroxides. From this finding, it was assumed that there might be heat stable stimulator of lipid peroxidation in addition to the heat labile inhibitor of lipid peroxidation which was found recently by us. EDTA completely prevented the inactivation of aniline hydroxylase and aminopyrine N-demethylase when rat liver 9000$\times g$ supernatant fraction was aged at 45°. When rat liver 9000$\times g$ supernatant fraction and microsomes were aged at 5° aerobically, activity of ethylmorphine N-demethylase was decreased and, inversely, amount of lipid peroxides formed during aging was increased. EDTA effectively inhibited the lipid peroxidation and decrease in ethylmorphine N-demethylase activity. The close relationship between lipid peroxidation and activity of ethylmorphine N-demethylase was confirmed using NADPH-generating system and ferrous ion as stimulators of lipid peroxidation.

Introduction

It has been well known that drug metabolizing enzymes in liver microsomes are exceedingly labile to the storage in various conditions. Recently, we[2] reported that aniline hydroxylase was kept rather stably when lyophilized microsomes were stored in anaerobic conditions, and that the enzyme in microsomes from rabbit liver was more stable than that in rat liver. There were several suggestions in the experiments. Microsomal membranes may undergo changes unfavourable to maintain activity of drug metabolizing enzymes in the presence of oxygen since the activity of aniline hydroxylase was decreased by an aerobic storage. In addition, the fact that aniline hydroxylase in rat liver microsomes was more labile than that in rabbit liver suggests the existence of a factor which catalyzes the degradation of microsomal membranes in the presence of oxygen in rat liver microsomes but not in rabbit liver microsomes. As regarding to the previous studies on the species difference in the stability of drug metabolizing enzymes, Gram and Fouts[3] demonstrated that rabbit liver enzymes were in most cases stable than mouse and rat liver enzymes, but failed to relate between the stability of drug metabolizing enzymes and microsomal lipid peroxidation.[4] Recently, we[5,6] have shown that there is an inverse correlation between the stability of drug metabolizing enzymes and microsomal lipid peroxidation using inhibitors and stimulators of lipid peroxidation, and have demonstrated[7] that there is the close relationship between ethylmorphine N-demethylating activity and lipid peroxidation in various species.

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1) Location: *1-33, Yayoi-cho, Chiba, 280, Japan.*
2) T. Kamataki and H. Kitagawa, *Jap. J. Pharmacol.*, "in press."
3) T.E. Gram and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **152**, 363 (1966).
4) T.E. Gram and J.R. Fouts, *Archs. Biochem. Biophys.*, **114**, 331 (1966).
5) T. Kamataki and H. Kitagawa, *Biochem. Pharmacol.*, **22**, 3199 (1973).
6) H. Kitagawa and T. Kamataki, Proc. 4th Symp. on Drug Metabolism and Action, Sendai, Sept. 1972, p. 83.
7) T. Kamataki and H. Kitagawa, *Biochem. Pharmacol.*, "in press."

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This investigation was initiated in order to further clarify the effects of ethylenediamine tetraacetic acid (EDTA), a potent inhibitor of lipid peroxidation, on the stability of drug metabolizing enzymes in various conditions.

Experimental

Male rats of Wistar strain (body weight is given in the Figures) were fasted for about 18 hr prior to sacrifice, but were given water *ad libitum*. Microsomes and $9000 \times g$ supernatant fraction were prepared by the method as previously described.[8] Experimental details on the incubation mixture are given for the Figures. NADPH-generating system consisted of NADP (0.33 mM, final concentration in the incubation mixture), glucose 6-phosphate (8 mM), $MgCl_2$ (6 mM) and 0.113 unit glucose 6-phosphate dehydrogenase. Oxidative demethylase activities were assayed with ethylmorphine and aminopyrine as substrates and the amount of formaldehyde formed was measured by the Nash reaction.[9] Aromatic hydroxylation of aniline was determined by measuring *p*-aminophenol according to the method of Kato and Gillette.[10] Lipid peroxide was determined as previously described.[5] The formation of lipid peroxides was represented as TBA values by calculating OD_{532nm} per microsomal protein (mg) added to the incubation mixture. The microsomal protein was determined according to the method of Lowry, *et al.*[11]

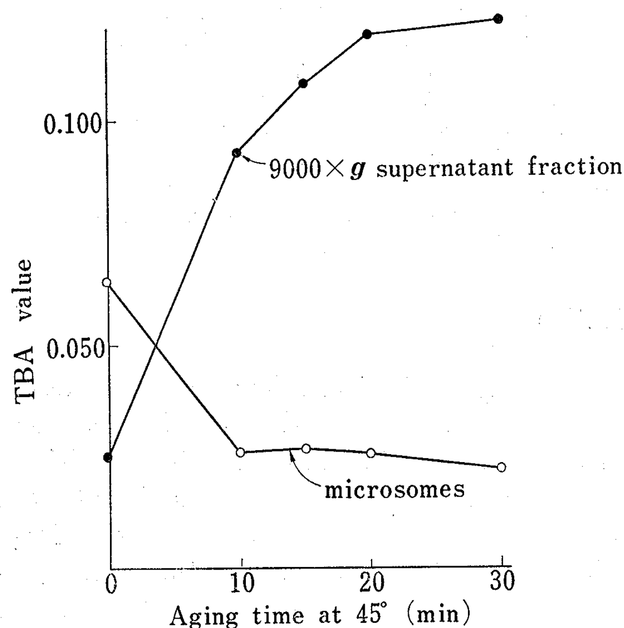

Fig. 1. Effects of Aging of Liver Microsomes or $9000 \times g$ Supernatant Fraction on Lipid Peroxidation

Livers of 6 rats weighing 84 to 110 g were pooled and used for the assay. After aging of microsomes or $9000 \times g$ supernatant fraction (2.43 mg of microsomal protein/ml) for each period, NADPH-generating system and 0.8 ml of 0.2M phosphate buffer (pH 7.4) were added to 1 ml of the aged samples, then incubated at 37° for 30 min.

Result and Discussion

1. Effects of Aging (at 45°) of Rat Liver $9000 \times g$ Supernatant Fraction and Microsomes on Lipid Peroxidation

When $9000 \times g$ supernatant fraction and microsomes were aged at 45° aerobically, the formation of lipid peroxides in microsomes was reduced with aging time, while unexpectedly, it was significantly increased in $9000 \times g$ supernatant fraction (Fig. 1). This fact may suggest that there is heat stable activator of lipid peroxidation in the soluble fraction.

2. Effects of EDTA on Activities of Aniline Hydroxylase and Aminopyrine N-Demethylase in $9000 \times g$ Supernatant Fraction Aged at 45°

To examine the effects of EDTA on aging-induced changes in drug oxidation rate, $9000 \times g$ supernatant fraction was aged at 45° aerobically with or without EDTA (2.5 mM). As shown in Fig. 2, aging of rat liver $9000 \times g$ supernatant fraction produced a marked decrease in aniline hydroxylation. As was expected, EDTA completely prevented the inactivation of aniline hydroxylase. The same experiment as Fig. 2 was performed using aminopyrine as a substrate but was added EDTA before or after the aging in order to compare the inactivation of the N-demethylase during aging (at 45°) and incubation periods (at 37°, 30 min). Fig. 3 represents stabilization of aminopyrine N-demethylase by EDTA both during aging period and

8) T. Kamataki, M. Shimokawa, and H. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 102 (1973).
9) T. Nash, *Biochem. J.*, **55**, 416 (1953).
10) R. Kato and J.R. Gillette, *J. Pharmacol. Exptl. Therap.*, **150**, 279 (1965).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

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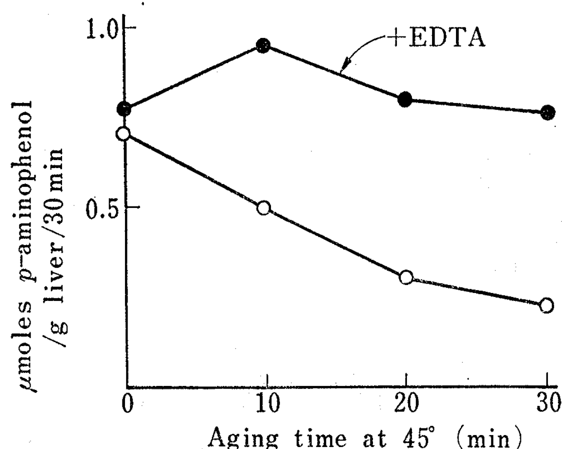

Fig. 2. Effect of EDTA on Aging-Induced Change in Aniline Hydroxylation

Livers of 6 rats (body weight average 154 g) were pooled and used for the assay. EDTA (final 2.5 mM) was added before aging. After aging of $9000 \times g$ supernatant fraction of liver at 45° aerobically, the substrate and co-factors were added and incubated at 37° for 30 min. The incubation system consisted of aniline (1 mM), NADPH-generating system, liver $9000 \times g$ supernatant fraction (1 ml, equivalent to 250 mg of liver) and 1 ml of 0.2 M Na-K phosphate buffer (pH 7.4) in a final volume of 2.5 ml.

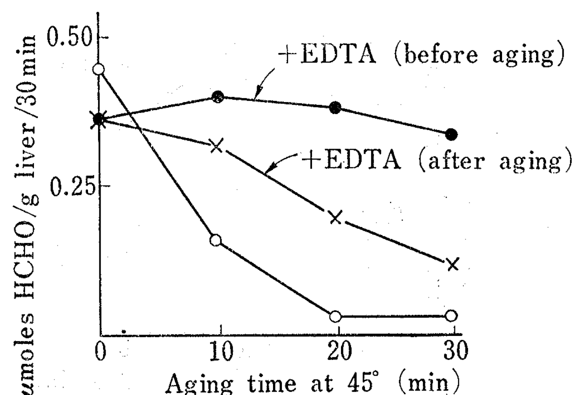

Fig. 3. Effect of EDTA on Aging-Induced Change in Aminopyrine N-Demethylation

Livers of 7 rats (body weight average 114 g) were pooled and used for the assay. EDTA was added before or after aging. Other experimental conditions were as described in Fig. 2 except that aminopyrine (1 mM) was used as a substrate instead of aniline.

incubation period. In this experiment, aminopyrine N-demethylation before aging at 45° was inhibited by EDTA. The inhibition may be caused since rather high concentration of EDTA was employed to inhibit aging-induced stimulation of lipid peroxidation which is shown in Fig. 1.

3. Effects of Aging (at 5°) of Rat Liver $9000 \times g$ Supernatant Fraction or Microsomes on Activity of Ethylmorphine N-Demethylase and Lipid Peroxidation

Effects of aging (at 5°) of rat liver $9000 \times g$ supernatant fraction on activity of ethylmorphine N-demethylase and lipid peroxidation were studied (Fig. 4). Lipid peroxidation was increased gradually with aging time. EDTA inhibited lipid peroxidation almost completely. Activity of ethylmorphine N-demethylase was decreased with increasing aging period, whereas the addition of EDTA resulted in stabilizing ethylmorphine N-demethylase. The inverse relationship between the activity of ethylmorphine N-demethylase and lipid peroxidation was essentially in agreement with the data described previously.[5] The same experiment as shown in Fig. 4 was performed using microsomes instead of $9000 \times g$ supernatant fraction (Fig. 5).

EDTA (0.1 mM) could not prevent lipid peroxidation during aging completely when aging time was prolonged. Then, the concomitant decrease in activity of ethylmorphine N-demethylase was observed. However, the effects of EDTA on lipid peroxidation and the activity of ethylmorphine N-demethylase were clearly shown during first 40 hr. These data presented in Fig. 4 and Fig. 5 may also suggest the existence of an inhibitor of lipid peroxidation in soluble fraction which have been recently characterized to be heat labile and to be induced by treatment of rats with 3-methylcholanthrene.[12,13]

12) T. Kamataki, H. Kaneda, K. Hara, and H. Kitagawa, Proc. General Meeting of Jap. Pharmacol. Soc., Sendai, April, 1972, p. 58.
13) T. Kamataki, N. Ozawa, M. Kitada, H. Kitagawa, and R. Sato, "in preparation."

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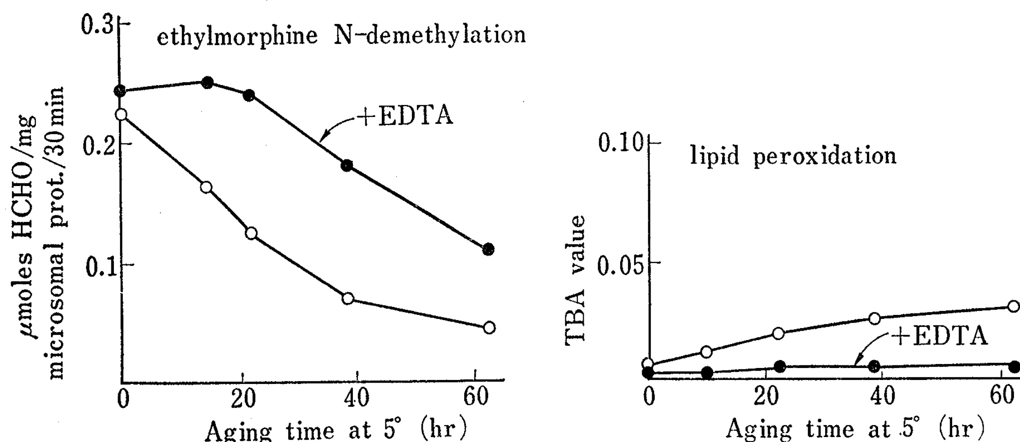

Fig. 4. Effects of EDTA on Aging-Induced Changes in Ethylmorphine N-Demethylation and Lipid Peroxidation in $9000 \times g$ Supernatant Fraction of Rat Liver

Eleven male rats weighing 110 to 150 g were used. All livers were pooled and used for enzyme assays listed in Fig. 4 to Fig. 7. Liver $9000 \times g$ supernatant fraction was aged at 5° aerobically with or without EDTA (final 0.23 mM). After aging, EDTA (final 0.1 mM) was added to prevent further occurence of lipid peroxidation. All vessels were incubated for measuring ethylmorphine N-demethylating activity after aging. Amount of lipid peroxides was measured before and after the incubation, whereas no differences in the TBA values between before and after incubation were observed. Incubation mixture consisted of ethylmorphine (final 1 mM), $9000 \times g$ supernatant fraction (1 ml, equivalent to 250 mg of liver), EDTA (final 0.1 mM), NADPH-generating system and 0.8 ml of 0.2 M Na–K phosphate buffer (pH 7.4) in a final volume of 2.5 ml. The NADPH-generating system and the substrate were added just before incubation at 37° for 30 min aerobically.

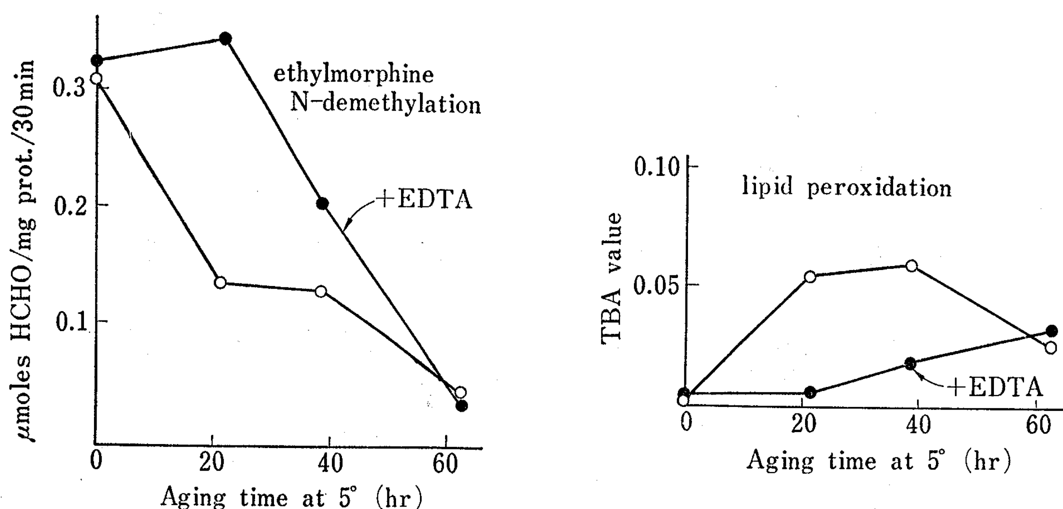

Fig. 5. Effects of EDTA on Aging-Induced Changes in Ethylmorphine N-Demethylation and Lipid Peroxidation in Microsomes of Rat Liver

All experimental procedures were as described for Fig. 4 except that microsomes (2.81 mg prot.) were employed instead of $9000 \times g$ supernatant fraction.

Lipid peroxidation is known to be stimulated by ferrous ion, ascorbic acid and NADPH. Then, the effects of EDTA on aging-induced changes in lipid peroxidation and ethylmorphine N-demethylase were studied in the presence of NADPH-generating system (Fig. 6). Lipid peroxidation was markedly stimulated by the addition of NADPH-generating system and it was strongly inhibited by EDTA. On the other hand, the activity of ethylmorphine N-demethylase was reduced to almost negligible level during the first 15 hr of aging period. EDTA effectively acted as a stabilizer of ethylmorphine N-demethylase.

As described above, ferrous ion and NADPH are stimulators of lipid peroxidation. The effects of NADPH-generating system on lipid peroxidation and the activity of ethylmorphine N-demethylase were tested in the presence of ferrous ion (Fig. 7). The addition of ferrous

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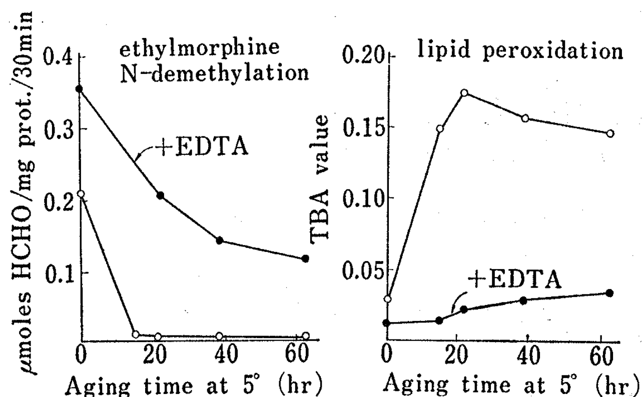

Fig. 6. Effects of EDTA on Aging of Rat Liver Microsomes with NADPH-Generating System

Microsomes were aged with NADPH-generating system and with or without EDTA. Other experimental conditions were as described for Fig. 5.

ion to the incubation mixture resulted in increase in lipid peroxidation and concomitant decrease in activity of ethylmorphine N-demethylase. It seems possible to confirm that the stimulation of lipid peroxidation produces the inactivation of ethylmorphine N-demethylase, and the inhibition of lipid peroxidation results in stabilizing ethylmorphine N-demethylase. Since the content of cytochrome P-450 and activities of NADPH-cytochrome c reductase and NADPH-neotetrazolium diaphorase were not decreased so much as activity of ethylmorphine N-demethylase when lipid peroxidation was stimulated with ferrous ion or ascorbic acid,[14] it may not be reasonable to assume that the inactivation of ethylmorphine N-demethylase is depending upon decrease in either cytochrome P-450 content or activities of NADPH-linked reductases.

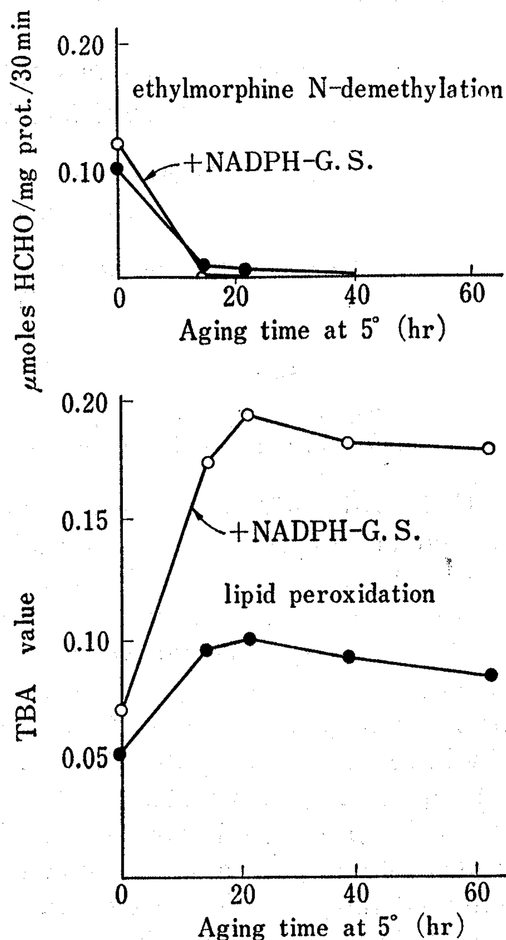

Fig. 7. Effects of NADPH-Generating System on Aging of Rat Liver Microsomes with Fe^{++}

Microsomes were aged at 5° aerobically with Fe^{++} (final 0.1 mM, as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) and with or without NADPH-generating system. Just after aging, EDTA (final 0.2 mM) and NADPH-generating system were added into all vessels. Other experimental conditions were as described for Fig. 5.

Acknowledgement We thank Miss Kuniko Hara for her skillful technical assistance.

14) M. Kitada, T. Kamataki, and H. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 752 (1974).

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