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Effects of Lipid Peroxidation on the Microsomal Electron Transport System and the Rate of Drug Metabolism in Rat Liver

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The effects of preincubation of microsomes with ascorbate or ferrous ion or NADPH-generating system on activities of drug metabolizing enzymes, content of cytochrome P-450 and the activities of NADPH-linked cytochrome c reductase and neotetrazolium diaphorase were investigated. Demethylase activities of aminopyrine, ethylmorphine and codeine were markedly decreased by the stimulation of microsomal lipid peroxidation whereas hydroxylation of aniline was only slightly reduced. On the other hand, content of cytochrome P-450 and activities of NADPH–cytochrome c reductase and NADPH–neotetrazolium diaphorase were only slightly changed by the stimulation of microsomal lipid peroxidation. Aminopyrine N-demethylation was inhibited by carbon monoxide but lipid peroxidation was increased. Furthermore, synergism of NADPH-dependent drug oxidation by NADH which has been reported by Estabrook, *et al.* was not observed in lipid peroxidation.

Introduction

Formation of lipid peroxides in liver microsomes has been known to produce the decomposition of microsomal membranes.[2,3] In addition, there are some reports showing that phospholipids are necessary for drug metabolizing enzymes and cytochrome P-450 reduction.[4–11] Wills[12] demonstrated that the stimulation of lipid peroxidation by addition of ascorbic acid or NADPH or by treatment of microsomes with ionizing radiation caused a sharp decrease in aniline hydroxylase activity and aminopyrine N-demethylase activity, and suggested that the decrease in activities of drug metabolizing enzymes was not caused by the toxic product formed by lipid peroxidation but by the disintegration of microsomal membranes because the addition of the product of lipid peroxidation, malonaldehyde, did not affect the activity of aminopyrine N-demethylase. In relation to the changes in the drug metabolizing enzymes, Schacter, *et al.*[13] have recently reported that cytochrome P-450 was rapidly decomposed by the stimulation of lipid peroxidation. It has been supposed by several investigators[12,14,15] that the activation of drug metabolizing enzymes should occur if lipid

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peroxidation is inhibited since integrity of microsomal membranes might be maintained by inhibition of lipid peroxidation. However, no successful results have yet been reported except in an aldrin epoxidase.[16] Recently, Kamataki and Kitagawa[17,18] have demonstrated the close relationship between lipid peroxidation and activities of drug metabolizing enzymes. Namely, the inhibition of lipid peroxidation by the addition of EDTA in the incubation mixture produced marked increase in activities of ethylmorphine, aminopyrine and codeine demethylases. Then, the present study was initiated in order to clarify whether inactivation of drug metabolizing enzymes by lipid peroxidation correlates with changes in content of cytochrome P-450 and activities of NADPH-linked cytochrome c reductase and neotetrazolium diaphorase.

Experimental

Male rats of Wistar strain weighing 90 to 140 g were maintained on a commercial rat chow, CE-2, Nippon Crea Co., LTD., and were fasted for about 18 hr prior to sacrifice but were given water *ad libitum*. Liver microsomes were prepared as reported previously.[19] Microsomal protein was determined according to the method of Lowry, *et al.*[20] Aniline hydroxylase activity was measured by determining the amount of *p*-aminophenol formed with a method of Brodie and Axelrod[21] as modified by Kato and Gillette.[22] Oxidative demethylase activities were assayed with aminopyrine, codeine and ethylmorphine as substrates and the amount of formaldehyde formed was measured by Nash reaction.[23] Cytochrome P-450 was determined by the method of Omura and Sato.[24] An extinction coefficient increment of 91 mM^{-1} cm^{-1} was used for calculation of cytochrome P-450 content from absorbance difference between 450 and 490 mμ. Activities of NADPH–cytochrome c reducase and NADPH–neotetrazolium diaphorase were assayed by the method of Williams and Kamin.[25] Lipid peroxidation was measured by determining malonaldehyde formed with the thiobarbituric acid (TBA) reaction.[17] The formation of lipid peroxides was shown as TBA values which were obtained by calculating OD_{532} mμ per microsomal protein added to the incubation mixture (mg). Description for the preparation of the incubation system is given in the Figures and Tables.

Result and Discussion

Effects of preincubation of microsomes with ascorbic acid on the activities of aniline hydroxylase and ethylmorphine N-demethylase were investigated (Fig. 1). Lipid peroxidation was stimulated by addition of ascorbic acid and was continuously increased during the period of preincubation. The stimulation of lipid peroxidation by ascorbic acid resulted in a marked decrease in ethylmorphine N-demethylase activity but in a slight decrease in activity of aniline hydroxylase.

As shown in Fig. 2, it is clear that N-demethylase activity of ethylmorphine is more decreased than hydroxylase activity of aniline even if the activities are represented either as activity/cytochrome P-450 or as activity/mg of microsomal protein. The reason why aniline hydroxylase is more stable to the alterations of microsomal membranes induced by lipid peroxidation is not as yet clear. However, it seems possible to assume that N-demethylase activity of ethylmorphine, type I compound, is more dependent with the lipid of microsomal membrane than hydroxylase activity of aniline, type II compound. Recently, Chaplin and Mannering[4] and Kamataki, *et al.*[19] have demonstrated that type I binding site is different from type II binding site in the dependency for phospholipids, and type I binding site is more dependent on the phospholipids of microsomal membranes than type II binding

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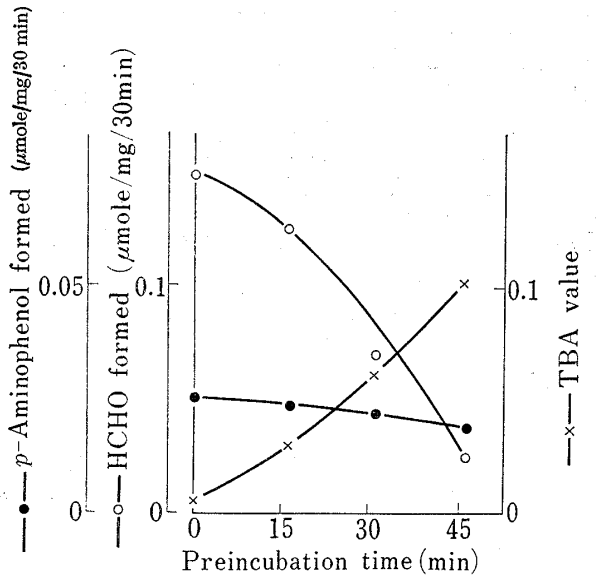

Fig. 1. Effects of Preincubation of Microsomes with Ascorbic Acid on Aniline Hydroxylation, Ethylmorphine N-Demethylation and Lipid Peroxidation

Microsomes (2.38 mg/ml) were preincubated with ascorbic acid (1 mM) at 37° aerobically for varying period as indicated in the Figure. After preincubation, NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 6 mM $MgCl_2$ and excess amount of glucose-6-phosphate dehydrogenase), substrate (1 mM), 0.8 ml of 0.2M phosphate buffer pH 7.4 and EDTA (0.1 mM) to inhibit further lipid peroxidation were added to one ml of microsomes in a final volume of 2.5 ml and were incubated for an additional 30 minutes at 37°.

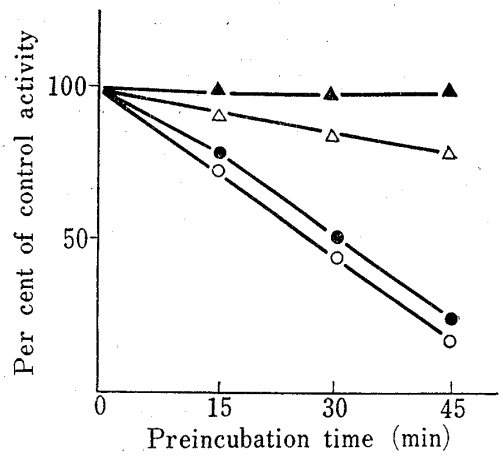

Fig. 2. Effects of Preincubation of Microsomes with Ascorbic Acid on Aniline Hydroxylation and Ethylmorphine N-Demethylation

Experimental details were the same as described for Fig. 1. The changes in the activities were shown as *p*-aminophenol formed/cytochrome P-450/30 min: —▲—, *p*-aminophenol formed/mg of microsomal protein/30 min: —△—, HCHO formed/cytochrome P-450/30 min: —●—, and HCHO formed/mg of microsomal protein/30 min: —○—, and were represented as per cent of control activities.

TABLE I. Comparison of Stability of Drug Metabolizing Enzymes against Preincubation with NADPH-Generating System

Substrate	Before preincubation (mμmole/mg/30 min)	After preincubation (mμmole/mg/30 min)	Decrease (%)
Aniline hydroxylation	39.7	29.0	27.0
Aminopyrine N-demethylation	65.8	15.2	76.9
Codeine demethylation	118.8	10.6	91.1
Ethylmorphine N-demethylation	71.3	1.7	97.7

Microsomes (3.25 mg/ml) were preincubated for 20 minutes with NADPH-generating system at 37°, then incubation mixture supplemented with an additional NADPH-generating system, various substrates and EDTA (0.1 mM) to inhibit further lipid peroxidation, were incubated for an additional 30 minutes at 37°.

site. Then we investigated the stabilities of drug metabolizing enzymes against preincubation with NADPH-generating system using various substrates.

As presented in Table I, activities of codeine, ethylmorphine and aminopyrine demethylation are decreased by about 91%, 98% and 77% of control activities, respectively, whereas hydroxylase activity of aniline is decreased by only about 27% of control. The results confirm that aniline hydroxylase is more stable than demethylases of codeine, ethylmorphine and aminopyrine against lipid peroxidation. In this respect, our results were essentially in agreement with those reported by Chaplin and Mannering.[4] Recently, Schacter, *et al.*[13] have reported that the stimulation of lipid peroxidation in liver microsomes by ADP and $FeCl_3$ resulted in degradation of cytochrome P-450. For that reason, we investigated the changes in content of cytochrome P-450 by preincubation of microsomes in relation to the changes in activities of drug metabolizing enzymes. As shown in Table II, content of cytochrome P-450 was

Table II. Effect of Preincubation of Microsomes with Ascorbic Acid on the Content of Cytochrome P-450

Preincubation (min)	Cytochrome P-450 (mμmole/mg)	Cytochrome P-450 (after preinc. / before preinc.)	TBA value
0	0.790	1.00	0.001
15	0.785	0.99	0.023
30	0.716	0.91	0.060
45	0.596	0.75	0.100

Experimental details were the same as those of Fig. 1.

reduced in proportion to the period of preincubation. Moreover, we observed practically no degradation of cytochrome P-450 when preincubation of microsomes were carried out in the absence of ascorbic acid (data not shown). However, our results were somewhat different from those of Schacter, *et al.*[13] in the degree of the decrease of cytochrome P-450. We observed rather small decrease in cytochrome P-450 content compared to the data reported by Schacter, *et al.*[3] The decrease in content of cytochrome P-450 was, therefore, less than those of demethylase activities. Then it seems possible to assume that the inactivation of drug metabolizing enzymes by lipid peroxidation are not depending upon the degradation of cytochrome P-450. These results led us examine further the changes in the enzyme activities of NADPH-linked electron transport system by lipid peroxidation. Activities of NADPH–cytochrome c reductase and NADPH–neotetrazolium diaphorase were investigated for the purpose to clarify the effects of lipid peroxidation (Fig. 3). Microsomal lipid peroxidation was stimulated by ferrous ion and was increased with preincubation time, and activity of ethylmorphine N-demethylase was decreased with an inverse pattern. On the other hand, no changes in activities in NADPH-linked cytochrome c reductase and neotetrazolium diaphorase were observed. These results suggest that the decrease in activities of oxidative demethylases is owing neither to the inactivation of NADPH–cytochrome c reductase and NADPH–neotetrazolium diaphorase nor to the decrease in cytochrome P-450 content but is owing to the disintegration of microsomal membranes. It has been well known that phospholipids which are major components of mi crosomal membranes are decomposed by lipid peroxidation. In addition, Strobel, *et al.*[9] have demonstrated that phosphatidylcholine is required for drug hydroxylation and Coon, *et al.*[11] have reported on its role that it is necessary for the enzymatic reduction of cytochrome P-450. From the results described above, it is likely to assume that the decomposition of microsomal phospholipids and/or the damage of the enzymatic reduction of cytochrome P-450 cause the decrease in activities of the demethylases. However, this speculation is insufficient to explain the facts that demethylase ac-

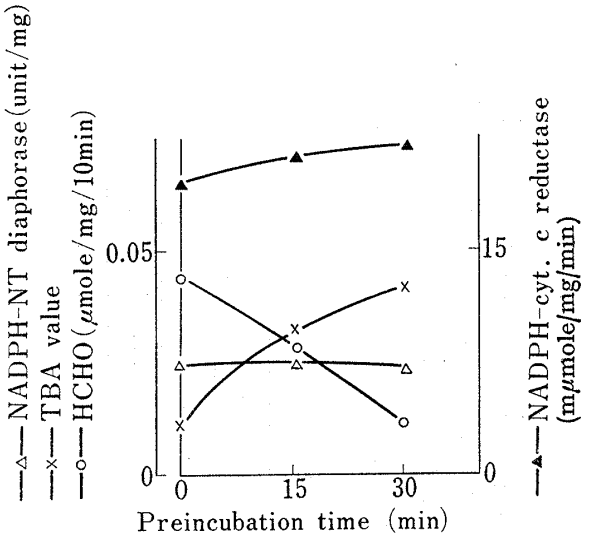

Fig. 3. Effects of Preincubation with Ferrous Ion on the Activities of NADPH–Neotetrazolium Diaphorase, NADPH–Cytochrome c Reductase and Ethylmorphine N-Demethylase

Microsomes (2.30 mg/ml) were preincubated with Fe^{2+} (20 μM, as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) at 37° for varying period as indicated in the figure and were used for the determination of NADPH–cytochrome c reductase and NADPH–neotetrazolium diaphorase activities. After preincubation, one ml of microsomes supplemented with NADPH-generating system, ethylmorphine (1 mM) and EDTA (0.1 mM) to inhibit further lipid peroxidation were incubated for an additional 10 minutes at 37°. The activity of NADPH–cytochrome c reductase was determined from the absorbance at 550 mμ using an extinction coefficient of 21.0 $cm^2 \mu mole^{-1}$.

tivities of aminopyrine, ethylmorphine and codeine were markedly decreased whereas activity of aniline hydroxylase was decreased only slightly. Therefore, it seems possible to explain by assuming that changes in drug metabolizing enzymes especially demethylases, produced by lipid peroxidation are induced from changes in interaction between cytochrome P-450 and phospholipids in microsomal membranes as discussed above.

Table III. Effects of Carbon Monoxide on Aminopyrine N-Demethylation and Lipid Peroxidation

	Gas phase	Aminopyrine N-demethylation (HCHO mμmole/mg/15 min)	TBA value/15 min
A	air	4.87 [1.00]	0.010 [1.00]
B	$CO:O_2:N_2=50:20:30$	4.09 [0.84][a)]	0.016 [1.60][a)]
C	$CO:O_2=80:20$	2.31 [0.47][a)]	0.023 [2.30][a)]

a) Numbers in blackets represent B/A and C/A respectively. Concentration of NADPH used in this experiment was 30 μM.

As described above, the authors have reported that N-demethylase activities of ethylmorphine and aminopyrine are increased by about 2-fold by addition of EDTA to the incubation mixture. We investigated the other relationships between lipid peroxidation and drug hydroxylation during the period of incubation. The effects of carbon monoxide on aminopyrine N-demethylation and lipid peroxidation are shown in Table III. Aminopyrine N-demethylation was inhibited by carbon monoxide whereas lipid peroxidation was evidently increased. The increment of lipid peroxidation by carbon monoxide suggests that lipid peroxidation reaction is not mediated through cytochrome P-450 though Hrycay and O'Brien[26,27] have suggested that lipid peroxidation can be catalyzed by cytochrome P-450 or cytochrome P-420. Recently, Cohen and Estabrook[28,29] demonstrated that the addition of NADH markedly increased N-demethylation of several drugs in the presence of NADPH. We examined whether the effect of NADH on the rate of ethylmorphine N-demethylation are affected by inhibition of lipid peroxidation. The results are given in Fig. 4. The addition of EDTA to the incubation mixture significantly increased apparent activity of ethylmorphine N-demethylase in all cases except in the case using NADH alone as an electron donor, and an additional effect of NADH on ethylmorphine N-demethylation was also occurred even in the presence of EDTA. These results suggest that the effect of NADH on ethylmorphine N-demethylation in the presence of NADPH is independent to lipid peroxidation.

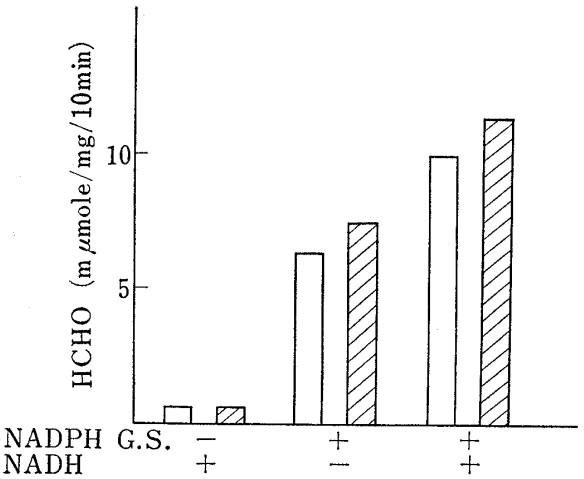

Fig. 4. Effect of EDTA on Ethylmorphine N-Demethylation in the Presence of NADPH-Generating System and/or NADH

Concentrations of NADH and EDTA used in this experiment were 1 mM and 0.1 mM respectively. Incubation was carried out at 37° for 10 minutes.
□ without EDTA, ▨ with EDTA

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