CAUSE OF DECREASE OF ETHYLMORPHINE N-DEMETHYLASE ACTIVITY BY LIPID PEROXIDATION IN MICROSOMES FROM THE RAT, GUINEA PIG AND RABBIT

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Abstract—There were marked differences among animal species between NADPH-dependent and ascorbic acid- Fe^{++} -dependent lipid peroxidation. In NADPH-dependent lipid peroxidation, this activity occurred to the greatest extent in rats followed by guinea pigs and rabbits and such was much lower in rabbits than in guinea pigs. On the other hand, rabbit microsomes exhibited higher lipid peroxidation activity than guinea pigs in ascorbic acid plus Fe^{++} or Fe^{++} -dependent lipid peroxidation although the activity was still lower than in rats. The ascorbic acid plus Fe^{++} -stimulated lipid peroxidation produced a decrease in ethylmorphine N-demethylase activity which was closely related to ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity but was not related to the change of the apparent content of cytochrome P-450 in all animal species. These results indicate that decrease of NADPH-cytochrome P-450 reductase activity induces a decrease in ethylmorphine N-demethylase activity by lipid peroxidation.

Unsaturated fatty acids in liver microsomes undergo peroxidative degradation. The lipid peroxidation reaction essentially requires ferrous ion and is stimulated by the addition of ascorbic acid or NADPH. As regards NADPH-dependent lipid peroxidation, NADPH-cytochrome *c* reductase has been demonstrated to be the enzyme which transfers electrons for the reaction (1). The NADPH-cytochrome *c* reductase also transfers electrons to cytochrome P-450, a drug hydroxylase. The close relationship between the apparent activities of drug metabolizing enzymes and lipid peroxidation has been established in our previous reports (2-5) and by others (6, 7). Schacter *et al.* (8) and Levin *et al.* (9) reported that microsomal lipid peroxidation results in a degradation of cytochrome P-450. This degradation is considered to be the cause of the decrease of drug metabolizing enzyme activity by lipid peroxidation. However, as we have already reported (10), when microsomes were preincubated with ascorbic acid for 45 min, the decrease of cytochrome P-450 content was at most 20-30% whereas the decrease of ethylmorphine N-demethylase activity was close to 90%.

This report concerns species differences among rats, guinea pigs and rabbits regarding lipid peroxidation stimulated by NADPH, ferrous ion and/or ascorbic acid. Also included are our findings on the relationship between lipid peroxidation stimulated by ferrous ion and/or ascorbic acid and the decrease in ethylmorphine N-demethylase activity, cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity.

MATERIALS AND METHODS

Male Wistar rats weighing 101 to 132 g, male guinea pigs weighing 260 to 430 g and male albino rabbits weighing 1.68 to 3.15 kg were maintained on commercial chow, and were starved for about 18 hr prior to sacrifice. Liver microsomes of the animals were prepared by a method previously described (11). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I) were purchased from Boehringer Mannheim Co. LTD. Thiobarbituric acid was purchased from Daiichi Pure Chemicals Co. LTD., Japan, and a 0.67 percent solution was prepared as described previously (3). Other reagents were from commercial sources and were used without further purification. NADPH-generating system contained NADP (0.33 mM), glucose 6-phosphate (8 mM), glucose 6-phosphate dehydrogenase (0.045 unit/ml of incubation mixture) and $MgCl_2$ (6 mM) in a final volume of 2.5 ml. Ethylmorphine N-demethylase activity was estimated by determining formaldehyde by the method of Nash (12). Lipid peroxides were determined by the thiobarbituric acid (TBA) method (3) and the lipid peroxidation activity was expressed in terms of thiobarbituric acid (TBA) value (5). Cytochrome P-450 content was determined by the method of Omura and Sato (13). NADPH-cytochrome P-450 reductase activity was measured essentially by the method of Diehl *et al.* (14) at 25°. All assays were performed in the presence of 80 mM Na, K-phosphate (pH 7.4). The microsomal protein was determined according to the method of Lowry *et al.* (15).

RESULTS

Gram and Fouts stated that rabbit liver 9,000 $\times$ g supernatant fraction (microsomes plus soluble supernatant) exhibited little lipid peroxidation as compared to that of the rat (16). More recently, we observed essentially the same species difference in microsomes. However, in contrast to the aforementioned data on the relationship between lipid peroxidation and drug metabolizing enzyme activity, we found that the inhibition of lipid peroxidation by EDTA resulted in a marked increase in the apparent hydroxylase activities of aniline, aminopyrine, codeine and ethylmorphine in rat liver microsomes (3). This data was later supported by Kotake *et al.* who used ethylmorphine as the substrate (7).

Figs. 1, 2 and 3 and Table 1 show the species differences of NADPH-dependent and ascorbic acid-dependent lipid peroxidation. These figures show the effects of EDTA and ferrous ion on NADPH-dependent lipid peroxidation and the apparent activity of ethylmorphine N-demethylase. These results indicate that rat liver microsomes have the highest activity for lipid peroxidation in both the presence and absence of external ferrous ion, followed by microsomes from guinea pigs and rabbits, and that EDTA almost completely inhibited lipid peroxidation in all animal species employed. The relationship between the time course of ethylmorphine N-demethylation and lipid peroxidation was also examined. In the presence of EDTA, the ethylmorphine N-demethylation was linear with incubation time. After addition of ferrous ion to the incubation mixture, ethylmorphine N-demethylation showed early deviation from the linear phase in the rat, but little effect was seen in the rabbit. Lipid peroxidation in the guinea pig microsomes was approximately half that

FIG. 1. Effects of ferrous ion and EDTA on lipid peroxidation and ethylmorphine N-demethylation in rat liver microsomes. The incubation mixture for the assay of lipid peroxidation and ethylmorphine N-demethylation contained an NADPH-generating system (see MATERIALS AND METHODS), microsomes (2.35 mg), Na, K-phosphate (80 mM, pH 7.4), ethylmorphine (1 mM) and either 0.1 mM EDTA or 20 μ M ferrous ion (as $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) in a final volume of 2.5 ml. The incubations were carried out at 37° for the varying periods shown. Each assay was carried out in duplicate on fresh microsomes pooled from ten rats.

FIG. 2. Effects of ferrous ion and EDTA on lipid peroxidation and ethylmorphine N-demethylation in guinea pig liver microsomes. Experimental details as in Fig. 1 except that microsomes added per incubation were 2.45 mg. Pooled microsomes from five guinea pigs were used for the assay.

FIG. 3. Effects of ferrous ion and EDTA on lipid peroxidation and ethylmorphine N-demethylation in rabbit liver microsomes. Experimental details as in Fig. 1 except that 1.15 mg microsomes were added per incubation.

TABLE 1. Species difference in microsomal lipid peroxidation stimulated by NADPH or ascorbic acid and/or ferrous ion

Animals	Additions	μ M	T B A value		
			Incubation time (min)		
			5	10	20
Rat	Fe^{++}	20	32.9 $\pm$ 5.5	33.7 $\pm$ 1.8	64.1 $\pm$ 0
		50	81.3 $\pm$ 23.2	65.8 $\pm$ 0.6	74.0 $\pm$ 0
		100	114.2 $\pm$ 9.3	112.6 $\pm$ 9.3	126.6 $\pm$ 1.1
	Ascorbic acid	50	22.2 $\pm$ 1.1	41.9 $\pm$ 1.1	72.3 $\pm$ 1.2
		100	28.8 $\pm$ 1.2	55.1 $\pm$ 1.8	100.3 $\pm$ 2.3
		300	37.0 $\pm$ 0	62.5 $\pm$ 1.8	129.0 $\pm$ 1.1
		500	33.7 $\pm$ 0.6	65.8 $\pm$ 0	127.4 $\pm$ 5.8
		1000	31.2 $\pm$ 0.6	55.9 $\pm$ 4.7	115.9 $\pm$ 4.7
	Fe^{++} (20 μ M) plus ascorbic acid	20	75.5 $\pm$ 4.4	99.3 $\pm$ 1.5	99.3 $\pm$ 1.4
		50	98.3 $\pm$ 8.1	138.6 $\pm$ 1.4	175.9 $\pm$ 22.2
		300	180.0 $\pm$ 0.7	273.1 $\pm$ 2.2	433.4 $\pm$ 12.4
	NADPH ^a	500	181.0 $\pm$ 5.1	369.3 $\pm$ 9.6	530.7 $\pm$ 4.4
		330	217.2 $\pm$ 7.3	364.1 $\pm$ 2.3	534.8 $\pm$ 9.5
Guinea pig	Fe^{++}	20	11.3 $\pm$ 0	16.7 $\pm$ 0	19.9 $\pm$ 1.3
		50	14.7 $\pm$ 0.3	24.4 $\pm$ 0.6	30.7 $\pm$ 4.5
		100	16.2 $\pm$ 0.3	32.5 $\pm$ 2.2	34.3 $\pm$ 2.6
	Ascorbic acid	100	8.1 $\pm$ 0.3	15.8 $\pm$ 0	29.8 $\pm$ 0.3
		300	9.5 $\pm$ 0.3	19.4 $\pm$ 1.6	36.5 $\pm$ 1.6
		500	8.1 $\pm$ 1.0	16.2 $\pm$ 0.3	31.6 $\pm$ 1.3
		1000	6.8 $\pm$ 0	11.7 $\pm$ 0.6	19.9 $\pm$ 1.0
	Fe^{++} (20 μ M) plus ascorbic acid	20	25.3 $\pm$ 5.7	35.1 $\pm$ 0.4	43.0 $\pm$ 0.4
		50	31.7 $\pm$ 1.0	53.8 $\pm$ 11.2	69.1 $\pm$ 5.2
		300	60.0 $\pm$ 0.6	112.7 $\pm$ 11.2	189.6 $\pm$ 12.0
	NADPH ^a	500	70.2 $\pm$ 1.6	134.7 $\pm$ 5.5	228.7 $\pm$ 2.6
		330	101.3 $\pm$ 2.5	228.1 $\pm$ 15.2	293.2 $\pm$ 6.0
Rabbit	Fe^{++}	20	14.8 $\pm$ 0	22.4 $\pm$ 4.0	42.7 $\pm$ 2.9
		50	39.7 $\pm$ 5.4	32.5 $\pm$ 5.8	59.0 $\pm$ 0.7
		100	55.4 $\pm$ 7.9	60.0 $\pm$ 1.8	61.0 $\pm$ 6.4
	Ascorbic acid	50	11.7 $\pm$ 0	19.3 $\pm$ 1.8	30.0 $\pm$ 2.5
		100	12.7 $\pm$ 0.4	22.4 $\pm$ 0	43.2 $\pm$ 2.6
		300	17.3 $\pm$ 1.4	30.0 $\pm$ 1.1	67.6 $\pm$ 2.1
		500	16.8 $\pm$ 0.4	32.0 $\pm$ 0.4	72.2 $\pm$ 2.6
		1000	15.3 $\pm$ 1.1	26.4 $\pm$ 0	51.9 $\pm$ 1.7
	Fe^{++} (20 μ M) plus ascorbic acid	20	40.9 $\pm$ 1.8	52.3 $\pm$ 11.6	62.6 $\pm$ 1.4
		50	54.9 $\pm$ 2.3	78.5 $\pm$ 2.7	84.3 $\pm$ 1.4
	NADPH ^a	330	39.6 $\pm$ 2.3	54.9 $\pm$ 3.6	83.0 $\pm$ 8.6

NADPH-dependent lipid peroxidation was assayed by a method essentially described for Fig. 1. Ascorbic acid and/or ferrous ion dependent lipid peroxidation was assayed using varying amounts of ascorbic acid and/or ferrous ion shown in the Table. The numbers given are the means $\pm$ SD of the means of duplicate determinations. ^a; NADPH was added as an NADPH generating system (see MATERIALS AND METHODS).

seen in the rat, however, the decrease of ethylmorphine N-demethylation was less than expected. Thus it may be assumed that ethylmorphine N-demethylase is more stable against lipid peroxidation in guinea pig than in rat liver microsomes.

To compare results of species differences in NADPH-dependent lipid peroxidation,

the lipid peroxidation stimulated by ferrous ion and/or ascorbic acid was measured in rat, guinea pig and rabbit microsomes. As shown in Table 1, rat microsomes exhibited higher lipid peroxidation activity in the presence of ferrous ion and/or ascorbic acid than did the other species. Of interest is the observation that guinea pig microsomes exhibited less lipid peroxidation activity than did those from the rabbit in the presence of ferrous ion and/or ascorbic acid. Actually, rabbit microsomes exhibited higher lipid peroxidation activity than guinea pig microsomes which is inverse as seen with the NADPH-dependent reaction. Ascorbic acid had a higher stimulative effect on lipid peroxidation in the presence of ferrous ion than did the additive activity from separate incubations with either ferrous ion or ascorbic acid.

The effects of preincubation of rat liver microsomes with ascorbic acid on ethylmorphine N-demethylase activity, cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity in the presence or absence of ethylmorphine are shown in Table 2. The control microsomes were also preincubated with ascorbic acid but with 0.1 mM EDTA to inhibit lipid peroxidation during the preincubation. The preincubation of microsomes with ascorbic acid produced a significant amount of lipid peroxides and a decrease of ethylmorphine N-demethylase activity. The decreases of the ethylmorphine N-demethylase activity

TABLE 2. Effects of preincubation of rat liver microsomes with ascorbic acid on activities of ethylmorphine N-demethylase and NADPH-cytochrome P-450 reductase and content of cytochrome P-450

Measurement	Preincubation condition		% Decrease
	- EDTA	--EDTA	
Ethylmorphine N-demethylation ^a	10.57	6.53	38.2
Cytochrome P-450 content ^b	1.18	0.91	22.9
NADPH-cyt. P-450 reductase (- LM) ^c	10.40	6.59	36.6
NADPH-cyt. P-450 reductase (+ LM) ^d	17.70	10.90	38.4
NADPH-cyt. P-450 reductase (LM-enhanced) ^e	7.30	4.31	41.0

Microsomes (2.1 mg protein) were preincubated with ascorbic acid (330 μ M) in the presence or absence of EDTA (0.1 mM) at 37° aerobically for 10 min. After the preincubation, EDTA (0.1 mM) was added to the preincubated medium without EDTA to inhibit further lipid peroxidation. The preincubated microsomes were used for the assay of ethylmorphine N-demethylase activity, NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content. Incubation time for the assay of ethylmorphine N-demethylase was 6 min and the substrate concentration was 1 mM. TBA values after preincubation for 10 min with ascorbic acid and EDTA and with ascorbic acid alone were 0.095 and 0.657, respectively. Each value is the mean of two separate determinations. *a*: Ethylmorphine N-demethylase activity, nmole formaldehyde formed/mg of protein/min incubation. *b*: Cytochrome P-450 content, nmole/mg of protein. *c*: NADPH-cytochrome P-450 reductase activity in the absence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *d*: NADPH-cytochrome P-450 reductase activity in the presence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *e*: Ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity, nmole cytochrome P-450 reduced/mg of protein/min.

TABLE 3. Effects of preincubation of guinea pig liver microsomes with ascorbic acid and ferrous ion on activities of ethylmorphine N-demethylase and NADPH-cytochrome P-450 reductase and content of cytochrome P-450

Measurement	Preincubation condition		% Decrease
	+EDTA	-EDTA	
Ethylmorphine N-demethylation ^a	5.31	4.31	18.8
Cytochrome P-450 content ^b	1.66	1.46	12.0
NADPH-cyt. P-450 reductase (- EM) ^c	8.24	6.81	17.4
NADPH-cyt. P-450 reductase (+ EM) ^d	11.87	9.75	17.9
NADPH-cyt. P-450 reductase (EM-enhanced) ^e	3.63	2.94	19.0

Microsomes (3.6 mg protein) were preincubated with ascorbic acid (300 μ M) and ferrous ion (20 μ M, as $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) in the presence or absence of EDTA (0.1 mM) aerobically for 10 min. Other experimental details are the same as for Table 2. TBA values after 10 min preincubation with ascorbic acid, ferrous ion and EDTA and with ascorbic acid plus ferrous ion were 0.085 and 0.725, respectively. Each value is mean of two separate determinations. *a*; Ethylmorphine N-demethylase activity, nmole formaldehyde formed/mg of protein/min incubation. *b*; Cytochrome P-450 content, nmole/mg of protein. *c*; NADPH-cytochrome P-450 reductase activity in the absence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *d*; NADPH-cytochrome P-450 reductase activity in the presence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *e*; Ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity, nmole cytochrome P-450 reduced/mg of protein/min.

TABLE 4. Effects of preincubation of rabbit liver microsomes with ascorbic acid and ferrous ion on activities of ethylmorphine N-demethylase and NADPH-cytochrome P-450 reductase and content of cytochrome P-450

Measurement	Preincubation condition		% Decrease
	+EDTA	-EDTA	
Ethylmorphine N-demethylation ^a	1.97	0.99	49.7
Cytochrome P-450 content ^b	0.96	0.72	25.0
NADPH-cyt. P-450 reductase (- EM) ^c	5.44	3.04	44.1
NADPH-cyt. P-450 reductase (+ EM) ^d	7.50	4.03	46.3
NADPH-cyt. P-450 reductase (EM-enhanced) ^e	2.06	0.99	51.9

Experimental details were the same as for Table 3 except that microsomes used were 2.4 mg protein per assay. TBA values after the preincubation for 10 min with ascorbic acid, ferrous ion and EDTA and with ascorbic acid and ferrous ion were 0 and 0.949, respectively. Each value is mean of two separate determinations. *a*; Ethylmorphine N-demethylase activity, nmole formaldehyde formed/mg of protein/min incubation. *b*; Cytochrome P-450 content, nmole/mg of protein. *c*; NADPH-cytochrome P-450 reductase activity in the absence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *d*; NADPH-cytochrome P-450 reductase activity in the presence of ethylmorphine, nmole cytochrome P-450 reduced/mg of protein/min. *e*; Ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity, nmole cytochrome P-450 reduced/mg of protein/min.

and cytochrome P-450 content were 38.2% and 22.9%, respectively. Calculating the difference in NADPH-cytochrome P-450 reductase activity in the presence and absence of ethylmorphine (ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity), it was shown that the decrease of ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity was 41.0%. Thus, the decrease of ethylmorphine N-demethylase activity by lipid peroxidation was most closely related to the change of ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity. Similar experiments have been conducted using guinea pig and rabbit liver microsomes. These microsomes were preincubated with ascorbic acid and ferrous ion to produce a higher amount of lipid peroxides. Control microsomes were preincubated under the same conditions but with 0.1 mM EDTA. As can be seen in Tables 3 and 4, in guinea pig and rabbit microsomes, the decrease of cytochrome P-450 content was much less than the decrease of ethylmorphine N-demethylase activity. A close relationship was always seen between the change in ethylmorphine N-demethylase activity and the change in NADPH-cytochrome P-450 reductase activity.

DISCUSSION

The decrease of the drug metabolizing enzyme activities has been accounted for by the decrease of cytochrome P-450 content by lipid peroxidation (6, 9). However, as we reported previously (10), it is obvious that the decrease of ethylmorphine N-demethylase activity by lipid peroxidation is much greater than that of cytochrome P-450 content although aniline hydroxylation activity decreased in parallel with cytochrome P-450. Since the apparent activity of NADPH-cytochrome *c* reductase was not decreased by lipid peroxidation (10), the cause of the decrease of ethylmorphine N-demethylase activity was not assumed to be due to the decrease of NADPH-cytochrome *c* reductase. Gigon *et al.* (17) have demonstrated that NADPH-cytochrome P-450 reductase activity is the rate limiting step for ethylmorphine N-demethylase activity. The NADPH-cytochrome P-450 reductase activity is assumed to be highly depending upon phospholipids as shown by Strobel *et al.* (18) and Coon *et al.* (19) who used partially purified NADPH-cytochrome *c* reductase and cytochrome P-450. Therefore, it seemed possible to assume that the decrease of ethylmorphine N-demethylase activity caused by lipid peroxidation could be accounted for by the decrease of NADPH-cytochrome P-450 reductase. In accordance with our hypothesis, the decrease of ethylmorphine N-demethylase activity was most closely related to the change of ethylmorphine-enhanced NADPH-cytochrome P-450 reductase activity. Therefore, the decrease of ethylmorphine N-demethylase activity by lipid peroxidation is most likely to be caused by degradation of lipids required for electron transfer from NADPH-cytochrome P-450 reductase to cytochrome P-450. Recent studies have established that hepatic microsomes contain multiple species of cytochrome P-450 (20–23). The possibility that lipid peroxidation caused degradation of a particular species of cytochrome P-450 which is specific for ethylmorphine N-demethylation cannot be ruled out.

The content of cytochrome P-450 was determined by the method of Omura and Sato (13) before and after preincubation of microsomes. Determination of cytochrome P-450

levels has been carried out with much care by several authors (6, 8, 9) since lipid peroxidation is considered the cause of degradation of the heme forming carbon monoxide. However, no detectable 450 nm peak was seen when reduced minus oxidized difference spectrum of lipid peroxidation-induced microsomes was recorded. Actually, a greater decrease of the apparent content of cytochrome P-450 would be expected if a considerable amount of carbon monoxide present in the incubation mixture was contained in the reference cuvette. A possible explanation may be that we used ascorbic acid and/or ferrous ion rather than NADPH-generating system to induce lipid peroxidation.

Acetanilide is non-enzymatically hydroxylated if ferrous ion, EDTA and molecular oxygen (Udenfriend's system) or ferrous ion, EDTA, ascorbic acid and hydrogen peroxide (Fenton system) (24) are present. Since ferrous ion, EDTA and ascorbic acid were present in our incubation mixture for the assay of ethylmorphine N-demethylation and since hydrogen peroxide may be produced by a NADPH-linked electron transport system (25), it is likely that the ethylmorphine N-demethylation activity of microsomes contained non-enzymatic formaldehyde formation. However, in the concentrations employed herein, there was no evidence of the non-enzymatic formation of formaldehyde due to the presence of ferrous ion, ascorbic acid and EDTA.

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