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THE OCCURRENCE OF AN INHIBITOR OF LIPID PEROXIDATION IN RAT LIVER SOLUBLE FRACTION AND ITS EFFECT ON MICROSOMAL DRUG OXIDATIONS

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In view of recent reports indicating that phospholipids are functionally involved in hepatic microsomal drug oxidizing activities (1-4), it is reasonable to assume that peroxidative destruction of phospholipids leads to impairment of the drug oxidizing machinery of liver microsomes. It has actually been shown that stimulation of lipid peroxidation by means of NADPH, ascorbate, ferrous ion, or ionizing irradiation markedly decreased aminopyrine demethylase and aniline hydroxylase activities of liver microsomes (5). We have also reported that microsomal ethylmorphine demethylase activity could be enhanced significantly when lipid peroxidation was inhibited by EDTA, Co^{2+}, or o-phenanthroline (6,7). Wills (8) has further demonstrated that liver microsomes from iron-overloaded rats had a higher lipid peroxidation activity and a reduced capacity to demethylate aminopyrine and p-chloro-N-methylaniline. On the other hand, Kuntzman (9) has observed that addition of liver soluble fraction back to microsomes improved the linearity of time course of microsomal benzpyrene hydroxylation significantly. Moreover, considerable enhancement of liver microsomal drug oxidations by the soluble fraction has recently been reported (10-13). It was, therefore, of interest to examine if the stimulatory effect of the soluble fraction was due to inhibition of microsomal lipid per-

oxidation. This communication reports evidence that liver soluble fraction contains an inhibitor(s) of lipid peroxidation and that the activation of drug oxidations by the soluble fraction can be partly accounted for by the presence of this inhibitor.

Male Wistar rats, weighing 79 to 160 g, were fasted for 18 hr and then killed by decapitation. The livers were thoroughly perfused *in situ* with isotonic KCl solution. The 9,000 x g supernatant (microsomal plus soluble), microsomal, and soluble fractions of liver homogenates were prepared in 1.15 % KCl as described previously (6). Protein was determined by the method of Lowry *et al.* (14). The reaction mixture for lipid peroxidation assay (final volume, 2.5 ml) contained 64 mM phosphate buffer (pH 7.4), 0.33 mM $NADP^+$, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$, 0.113 unit glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Boehringer Mannheim, Grade I), and either microsomes, 9,000 x g supernatant fraction, or a mixture of microsomes and soluble fraction. The reaction was carried out at 37 °C for 30 min aerobically. The formation of lipid peroxides was determined as described previously (6) and expressed in terms of thiobarbituric acid (TBA) value (absorbance at 532 nm due to TBA formation/mg of microsomal protein per ml of reaction mixture). Oxidative demethylation of ethylmorphine and aminopyrine was measured by determining the amount of formaldehyde formed by the method of Nash (15) under the same experimental conditions as above except that the respective drug substrate (usually 1 mM) and 5 mM semicarbazide were added to the reaction mixture. Other experimental conditions are given in the tables and figure.

The presence of an inhibitor(s) of lipid peroxidation in the soluble fraction was first suggested by the observation that microsomes formed significantly more lipid peroxides in the presence of the NADPH-generating system than the 9,000 x g supernatant fraction containing approximately the same amount of microsomal protein (Table 1). A similar inhibition of lipid peroxidation was also observed when a mixture of microsomes and the soluble fraction was used instead of the 9,000 x g supernatant fraction. However, the extent of inhi-

bition varied considerably from one experiment to another. It seemed that unknown physiological factors were responsible for such variation; we could at least notice the presence of a seasonal variation of the inhibitory activity of the soluble fraction.

TABLE 1. Lipid Peroxide Formation in Microsomes and 9,000 x g Supernatant Fraction of Rat Liver

Preparation	TBA value/30 min
Microsomes	0.248 ± 0.015
9,000 x g sup.	0.150 ± 0.035*

Rats weighing 120 to 130 g were used. Microsomes and 9,000 x g supernatant fraction were prepared from the same livers. A portion of the supernatant fraction was centrifuged at 105,000 x g for 1 hr to determine the amount of microsomal protein contained in that fraction. The amount of microsomal protein present in each incubation mixture was adjusted to 3.7 ± 0.3 mg. The other experimental conditions are described in text. The TBA values represent mean ± S.E. (n=4). * Differs significantly ($p < 0.05$)

In confirmation of previous findings (10-13), addition of the soluble fraction (freshly prepared from control rats) to microsomes caused substantial increases in the microsomal activities to demethylate ethylmorphine and aminopyrine. To see if these increases in microsomal drug oxidizing activities were due to inhibition of lipid peroxidation by the soluble fraction, the effect of the soluble fraction was compared with that of 0.1 mM EDTA which has been shown to prevent lipid peroxidation almost completely (6). As shown in Table 2, addition of EDTA to microsomes resulted in more than 2-fold stimulation of the demethylase activities using 1 mM ethylmorphine and aminopyrine as substrates. When 10 mM aminopyrine was used as substrate, the stimulation was only about 1.3 fold. This seemed to be due to the fact that 10 mM aminopyrine itself was capable of strongly inhibiting lipid peroxidation. When both the soluble fraction and EDTA were added simultaneously to the incubation mixture, the demethylase activities were increased to levels which were higher than those

attainable upon separate addition of the soluble fraction and EDTA. However, the activities produced by the simultaneous addition were less than those expected from an additive effect. It seemed, therefore, likely that the soluble fraction contained an activator of drug oxidations other than the inhibitor of lipid peroxidation, although a considerable portion of the stimulatory action of the soluble fraction could be replaced by EDTA.

TABLE 2. Effects of EDTA and/or Soluble Fraction on Microsomal Ethylmorphine and Aminopyrine Demethylase Activities

Addition \ Substrate	Demethylation rate (nmoles/mg/30 min) Ethylmorphine (1 mM)	Aminopyrine (1 mM)	Aminopyrine (10 mM)
Microsomes	83.3 ± 3.0	37.7 ± 0.4	147.4 ± 1.1
+SF	228.1 ± 6.2*	98.3 ± 2.9*	249.3 ± 5.1*
+EDTA	201.6 ± 0.8*	82.0 ± 1.7*	193.4 ± 0.8*
+SF +EDTA	266.7 ± 4.4*	110.8 ± 3.2*	270.8 ± 3.7*

Rats weighing 79 to 109 g were used. Livers from 8 rats were pooled and used for preparation of microsomes and soluble fraction. The demethylase activities were measured as described in text using microsomes containing 2.0 to 2.6 mg of protein and indicated concentration of respective substrate. Where indicated, the soluble fraction (12.0 mg of protein), 0.1 mM EDTA, or both were added to the incubation mixture. * Differs significantly ($p < 0.001$). SF, soluble fraction.

Since NADH has been shown to stimulate NADPH-dependent drug oxidations by liver microsomes (16-19) and since liver soluble fraction contains both NAD and NADH (20), it seemed possible that NADH contained in the soluble fraction might be responsible for the extra activation effect which could not be replaced by EDTA. Figure 1 shows the results of an experiment designed to test this possibility. It can be seen that addition of 0.1 mM EDTA to microsomes not only increased the initial rate of ethylmorphine demethylation but also improved the linearity of the reaction. A similar improvement of the reaction kinetics was also effected by addition of the soluble fraction. When both EDTA and the sol-

uble fraction were added to the incubation mixture, a further increase in the demethylation rate was observed in agreement with the results shown in Table 2. On the other hand, NADH at 1 mM markedly increased the initial rate of demethylation as reported previously (16-19), but failed to prolong the linear phase of the reaction. However, when EDTA or the soluble fraction or both were added to the incubation mixture in addition to NADH, the reaction kinetics was improved to an extent similar to (but not more than) that attained by the combined action of EDTA and the soluble fraction in the absence of NADH.

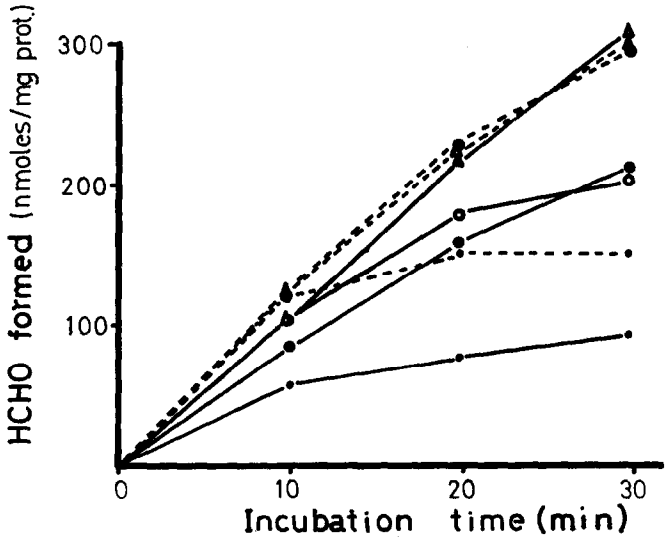

Fig. 1. Effects of EDTA and Soluble Fraction on Microsomal Ethylmorphine N-Demethylase Activity in the Presence and Absence of NADH

Rats weighing 140 to 160 g were used. Livers from 15 rats were pooled and used for preparation of microsomes and soluble fraction. The basic incubation mixture consisted of microsomes (1.94 mg of protein), 1 mM ethylmorphine, NADPH-generating system, and phosphate buffer (see text) in a final volume of 2.5 ml. When necessary, 0.1 mM EDTA, soluble fraction (7.2 mg of protein), and 1.0 mM NADH were added to the reaction mixture wither separately or in combination. •——• , control; ●——● , plus EDTA; ○——○ , plus soluble fraction (SF); ▲——▲ , plus EDTA and SF; •----• , plus NADH; ●---● , plus EDTA and NADH; ▲---▲ , plus EDTA,SF, and NADH.

Although further work is still needed, the results described in this paper seem to support the idea that liver soluble fraction contains a factor(s) capable of inhibiting microsomal lipid peroxidation and that the activation of microsomal drug oxidizing activities by the soluble fraction can be accounted for by this inhibitor of lipid peroxidation and another factor which can be replaced by NADH. A study on the chemical nature of the inhibitor is now in progress.

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