ETHANOL AND DRUG METABOLISM IN MOUSE LIVER MICROSOMES SUBSEQUENT TO LIPID PEROXIDATION-INDUCED DESTRUCTION OF CYTOCHROME P-450

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Received September 11,1974

Summary: Preincubation of mouse liver microsomes with NADPH resulted in malondialdehyde formation, destruction of cytochrome P-450, and decreased rates of aniline hydroxylation and N-demethylation of aminopyrine and ethylmorphine. These phenomena were more pronounced in phosphate than in Tris buffer. No reduction in rates of NADPH-linked oxidation of ethanol or in the activities of NADPH oxidase and NADPH-cytochrome c reductase was observed. While addition of EDTA to preincubation mixtures prevented lipid peroxidation, loss of cytochrome P-450, and inactivation of the drug-metabolizing capacity of microsomes, it did not alter ethanol oxidation rates and the activities of NADPH oxidase and NADPH-cytochrome c reductase. These findings argue against the involvement of cytochrome P-450 in the microsomal ethanol-oxidizing system.

Despite numerous reports that the NADPH-dependent microsomal metabolism of ethanol *in vitro* is mediated by the concerted action of NADPH oxidase and catalase (1-4), other investigations (5, 6) have argued for a distinct microsomal ethanol-oxidizing system (MEOS) that is "akin to other drug-metabolizing systems" (7) in which cytochrome P-450 serves as the terminal oxidase (8). Conflicting results with solubilized microsomal preparations have not resolved the controversy (9-12).

In the presence of NADPH and O_2, unsaturated fatty acids of liver microsomal phospholipids undergo peroxidation (13, 14), a process which destroys the architecture of microsomal membranes and leads to the formation of malondialdehyde (MDA). Levin *et al.* (15) have shown that incubation of microsomes with NADPH resulted in the breakdown of cytochrome P-450 heme, a parallel loss of cytochrome P-450, and in MDA formation. These processes could be blocked by EDTA, an inhibitor of lipid peroxidation.

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Present studies were undertaken to determine the effect of NADPH-induced lipid peroxidation on the capacity of microsomes to catalyze the NADPH-dependent oxidation of ethanol. After preincubation of microsomal suspensions with NADPH ± EDTA, MDA, cytochrome P-450, NADPH oxidase, NADPH-cytochrome c reductase, and ethanol- and drug-metabolizing activities were assayed and compared with unincubated microsomes as well as with microsomes incubated for 30 min in the absence of NADPH.

Methods

Livers from 15-20 adult male Swiss albino mice (25-27 g body weight) were pooled and microsomes prepared from 25% liver homogenates (15). The microsomal pellets were washed once with 1.15% KCl, layered either with 0.1 M K_2HPO_4-KH_2PO_4 (pH 7.4) or with 0.05 M Tris buffer (pH 7.5), and stored frozen overnight at -18°C (15, 16). Immediately before use, microsomes were thawed at room temperature and resuspended in the appropriate buffer to a concentration equivalent to 500 mg of liver (wet weight) per ml. Two milliliters of this suspension were subsequently incubated for 30 min at 37°C in a total volume of 18 ml of either buffer (1:18 w/v, 0.9 mg protein/ml) containing 0.5 mM NADPH or 0.5 mM NADPH and 1.0 mM EDTA. Control incubations consisted of similar suspensions that were either kept in ice or incubated for 30 min at 37°C in the absence of NADPH.

Cytochrome P-450 was determined by the method of Raj and Estabrook as described by Levin et al. (15). NADPH oxidase was assayed by the method of Gigon et al. (17), and NADPH-cytochrome c reductase as described by Sottocasa et al. (18). Rates of ethanol and drug metabolism were determined in incubation mixtures consisting of microsomes from 100 mg of liver (1.8 ml of the microsomal suspension; 1.6 mg of protein), 1.4 mM NADPH and substrate in a total volume of 2.0 ml of either 0.1 M phosphate (pH 7.4) or 0.05 M Tris buffer (pH 7.5). Final substrate concentrations were: ethanol,

88 mM; aniline and aminopyrine, 8.0 mM; and ethylmorphine, 4.0 mM. Incubations were carried out for 10 min at 37°C, except for aminopyrine which was for 5 min. Under these conditions, all enzymic reactions studied were linear both with respect to time and protein concentration. All incubations were performed in triplicate, with zero time determinations in duplicate. Ethanol oxidation was assayed by acetaldehyde formation (5). The p-hydroxylation of aniline and the N-demethylation of aminopyrine and ethylmorphine were estimated according to previously described methods (19, 20).

Results and Discussion

In accordance with the results of Levin et al. (15) obtained with rat liver microsomal suspensions, preincubation of mouse liver microsomes with NADPH in phosphate buffer led to a 55% decrease in cytochrome P-450 content (Fig. 1A). The breakdown of cytochrome P-450 was accompanied by the formation of 30 nmoles MDA/30 min/ml suspension as well as by a parallel decrease in the rate of aniline hydroxylation, a Type II substrate. A much more precipitous decrease in the rates of N-demethylation of the Type I substrates, aminopyrine and ethylmorphine, was obtained. It has been established that the reduction of a partially purified fraction of cytochrome P-450 by NADPH and purified reductase has an absolute requirement for lipid (21). Since the rate of reduction of cytochrome P-450 is the rate-limiting step in the metabolism of Type I but not Type II compounds (17, 22), the destruction of endogenous phospholipids via lipid peroxidation may retard the subsequent metabolism of Type I but not Type II drugs to a greater extent than the percent relative loss of cytochrome P-450.

The fall in rates of drug metabolism (Fig. 1A) is in agreement with the observations of Wills (23) who demonstrated that aminopyrine demethylation declined 50% following the induction of lipid peroxidation by preincubating rat liver microsomes with NADPH. Using similar methods for inducing lipid peroxidation, Kamataki and Kitagawa (24) demonstrated that codeine demethylase activity fell sharply with time to 5-10%

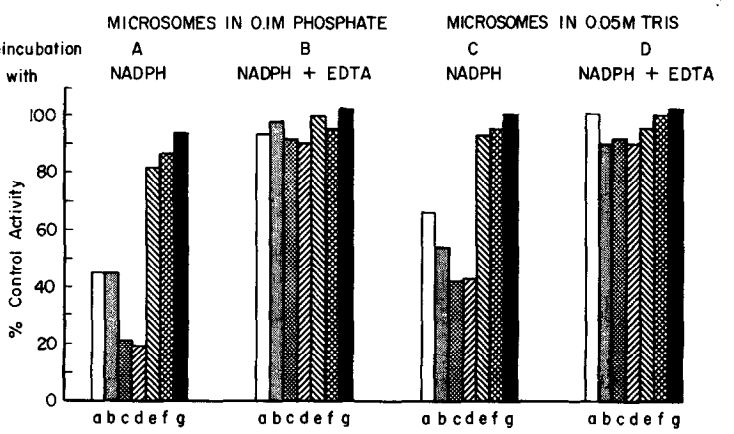

Figure 1. Effects of NADPH-induced lipid peroxidation on cytochrome P-450 content and on ethanol- and drug-metabolizing enzyme activities in mouse liver microsomes.

Microsomes equivalent to 1.0 g of liver were preincubated in air for 30 min at 37°C in a total volume of 18.0 ml (phosphate or Tris) containing either 0.5 mM NADPH (A, C) or 0.5 mM NADPH and 1.0 mM EDTA (B, D). After preincubation, aliquots were assayed as described in METHODS. Control levels (100%) (nmoles/min/mg protein for enzymic activities and nmoles/mg protein for cytochrome P-450) were obtained by preincubating microsomal suspensions for 30 min in the absence of NADPH. These were (for phosphate and Tris buffers, respectively): a. Cytochrome P-450, 1.23 and 1.06; b. Aniline p-hydroxylation, 0.9 and 1.2; c. Aminopyrine N-demethylation, 10.2 and 6.0; d. Ethylmorphine N-demethylation, 8.9 and 4.7; e. Ethanol oxidation, 11.9 and 8.1; f. NADPH oxidase, 7.8 and 5.0; and g. NADPH-cytochrome c reductase, 219 and 187. Values reported are the means of two separate experiments. These values were 80 to 90% of the zero-time (unincubated) controls.

of zero-time control levels, and this was accompanied by a parallel increase in MDA formation with time.

In contrast to the marked reduction in cytochrome P-450 levels and the even greater decrease in Type I substrate metabolism, preincubation of microsomes with NADPH resulted in only a slight decrease (18%) in ethanol oxidation. This can be explained by the parallel decrease in NADPH oxidase activity (Fig. 1A), since the rate of generation of H_2O_2 from NADPH and O_2 via NADPH oxidase is considered to be the rate-limiting step in the peroxidatic decomposition of ethanol by catalase (2, 3). The results shown in Fig. 1A confirm these latter findings. Although catalase was not assayed, it is doubtful that a decrease in its activity would have resulted from these

manipulations as only a small decrease in another hemoprotein, cytochrome b_5, was observed (15). Even if a portion of the catalase activity were lost, it would probably have had no detrimental effect on ethanol oxidation; a 99% reduction in catalase activity was accompanied by only a 40% decrease in acetaldehyde formation resulting from the NADPH-dependent microsomal ethanol oxidation (25). It may also be seen in Fig. 1A that no effect on the activity of NADPH-cytochrome c reductase was observed, confirming earlier findings that lipid is not required for maximal activity of this flavoprotein (21, 26).

As shown in Fig. 1B, EDTA, a known inhibitor of lipid peroxidation, protected against the NADPH-mediated destruction of cytochrome P-450, and the loss of drug-metabolizing activities. No MDA was formed under these conditions. In agreement with previous findings (16), much smaller rates of MDA formation (8.7 nmoles/30 min/ml suspension) and less destruction of cytochrome P-450 were observed in microsomes preincubated in Tris as opposed to phosphate buffer (compare Fig. 1A and 1C). As a consequence, drug-metabolizing activities were decreased to a lesser extent in Tris than in phosphate buffer. Nevertheless, a striking reduction in rates of drug metabolism was still obtained in Tris, and, as was the case with phosphate, losses in aniline hydroxylase activity paralleled the destruction of cytochrome P-450 much more closely than did the observed decrease in rates of aminopyrine and ethylmorphine demethylation (Fig. 1C). Again, no reduction in ethanol-oxidizing activity was observed nor were NADPH oxidase and NADPH-cytochrome c reductase activities altered (Fig. 1C). EDTA completely inhibited MDA formation, thus preventing the destruction of cytochrome P-450. Enzymic activities were at control levels (Fig. 1D).

It is evident from the results presented in this paper that the terminal oxidase of the microsomal NADPH-linked electron transport system does not participate in ethanol metabolism. The postulation of a MEOS that utilizes cytochrome P-450 by

interacting with the microsomal mixed-function oxidase "through competition for an at least partially common detoxifying system in the liver" (27) is unwarranted.

Acknowledgement

The authors are grateful to Mr. Michael Iba for his assistance in the spectral determinations of cytochrome P-450, as well as for his constructive criticisms.

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