MOLECULAR PHARMACOLOGY, 9, 670-675

Induction of Hepatic Microsomal Reduced Nicotinamide Adenine Dinucleotide Phosphate-Dependent Production of Hydrogen Peroxide by Chronic Prior Treatment with Ethanol

RONALD G. THURMAN

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19174

(Received March 21, 1973)

SUMMARY

THURMAN, RONALD G.: Induction of hepatic microsomal reduced nicotinamide adenine dinucleotide phosphate-dependent production of hydrogen peroxide by chronic prior treatment with ethanol. *Mol. Pharmacol.* **9**, 670–675 (1973).

Treatment of rats for 21–28 days with a semiliquid diet containing ethanol resulted in a near doubling of liver microsomal cytochrome P-450 content. Concomitantly, statistically significant increases in the rate of NADPH oxidation, "endogenous" respiration, and acetaldehyde formation from ethanol in microsomes were observed. An average increase in NADPH-dependent hydrogen peroxide formation of 45 ± 7% (SE) was observed as a result of chronic ethanol treatment, employing the decrease in scopoletin fluorescence or the formation of cytochrome peroxidase complex II as hydrogen peroxide-detecting systems. Since it has been reported that the rate-limiting step for ethanol oxidation in microsomes is the rate of generation of hydrogen peroxide for the peroxidatic reaction of catalase [R. G. Thurman, H. G. Ley, and R. Scholz, *Eur. J. Biochem.* **25**, 420–430 (1972)], this adaptive increase in hydrogen peroxide production due to chronic ethanol treatment most likely accounts for the enhanced ethanol oxidation via catalase-H_2O_2. The data are consistent with the hypothesis that microsomal ethanol oxidation is due to peroxidation via catalase utilizing microsomal hydrogen peroxide.

INTRODUCTION

The observation that the microsomal fraction of liver can oxidize ethanol to acetaldehyde in the presence of NADPH has generated considerable interest and controversy recently (1–4). This microsomal ethanol-oxidizing system has been characterized, primarily by Lieber and DeCarli (2), and shown to share certain characteristics with reactions of the classical mixed-function oxidase type. Its activity can be induced by prior treatment of the animal with ethanol (5). Another pathway by which ethanol can be oxidized, the peroxidatic reaction of ethanol with the catalase–hydrogen peroxide complex (4), has been demonstrated in microsomes. There are several characteristics common to microsomal ethanol oxidation and NADPH-dependent hydrogen peroxide production by microsomes. For example, both processes require NADPH, have identical oxygen dependence profiles, and are sensitive to inhibition by carbon monoxide (2, 4). In

This investigation was supported in part by Research Scientist Development Award K2-MH-70,155 and by Grant AA-00288 from the National Institute of Mental Health.

addition, microsomal ethanol oxidation can be inhibited by various procedures which are known to interact with the H_2O_2-catalase mechanism, such as (a) inhibition by substrates for catalase (formate, ascorbate), (b) inhibition by catalase inhibitors (azide), and (c) inhibition by H_2O_2-utilizing systems (4). Based on these observations Thurman, Ley, and Scholz have concluded that the postulation of a unique microsomal ethanol-oxidizing system in addition to the peroxidatic reaction of catalase is unnecessary (4). Opposing this point of view are reports that the activity of catalase is unaltered by chronic ethanol treatment (6), whereas the adaptive increase in the ability of the microsomal fraction to oxidize ethanol is well documented (2, 6, 7).

However, since the rate-limiting step for the H_2O_2-catalase mechanism in microsomes has been shown to be the rate of generation of H_2O_2 (4), changes in enzyme activity would be inconsequential to the over-all rate of ethanol oxidation. On the other hand, if a catalase-H_2O_2 mechanism were operative, then an increase in H_2O_2 production should accompany the increased rate of ethanol oxidation in microsomes associated with ethanol treatment, as is suggested from the observation that NADPH oxidase activity increase (6, 7). Thus this study was undertaken to examine the effect of chronic ethanol treatment on NADPH-dependent microsomal hydrogen peroxide production.

MATERIALS AND METHODS

Chronic ethanol treatment. Male Holtzman rats were fed a semiliquid diet modified slightly from that described by DeCarli and Lieber (8). The diet contained, as a percentage of total calories; carbohydrate, 46%; fat, 36%; and protein, 18%. A mixture of dextrin and maltose served as a source of carbohydrate; olive and corn oils, as the source of fat; and casein hydrolysate supplemented with essential amino acids, as the source of protein. When alcohol was employed, it replaced up to 36% of the total calories. The animals were housed in individual cages.

Microsomal preparation. Once-washed microsomes were prepared from liver essentially as described by Hildebrandt *et al.* (9). Protein was determined by the biuret reaction (10), employing appropriate controls for the turbidity of the microsomal suspension.

Acetaldehyde production from ethanol by microsomes. NADPH-dependent ethanol oxidation by the microsomal fraction was measured essentially as described previously (4). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 20 mM nicotinamide, 10 mM magnesium chloride, 50 mM ethanol, and an NADPH-generating system consisting of final concentrations of 0.4 mM $NADP^+$, 30 mM DL-isocitrate, and 200 milliunits/ml of isocitrate dehydrogenase (Sigma). Incubations were performed in capped 25-ml Erlenmeyer flasks with center wells containing semicarbazide (15 mM in 180 mM phosphate buffer, pH 7.0), in a shaking water bath at 37°. Reactions were initiated with microsomes (2–4 mg/ml of reaction mixture) and were terminated after 10 min by the addition of trichloracetic acid (final concentration, 0.6 M). The acetaldehyde produced during the incubation was trapped as the semicarbazone after an overnight diffusion period and compared with acetaldehyde standards at 224 nm (11). Incubations were performed in triplicate.

Microsomal NADPH-dependent hydrogen peroxide formation. The generation of H_2O_2 by NADPH and oxygen by microsomes was measured spectrophotometrically by the formation of cytochrome *c* peroxidase complex II (12), of fluorometrically by the decrease in scopoletin fluorescence in the presence of horseradish peroxidase (13). Scopoletin is a fluorescent coumarin derivative which is a substrate for horseradish peroxidase. In the presence of H_2O_2 and the enzyme it is converted into a nonfluorescent product, and the decrease in scopoletin fluorescence is an excellent assay system for H_2O_2 production.

Cytochrome P-450 concentrations. The microsomal content of cytochrome P-450 was determined by the absorption differences between the sodium dithionite-reduced and the (reduced + carbon monoxide) pigment in an Aminco double-beam spectrophotometer. Although the molar extinction coeffi-

cient of cytochrome P-450 remains controversial, the number (91 mM^{-1} cm^{-1}) reported by Omura and Sato (14) was used to calculate concentrations in these experiments.

Endogenous respiration and NADPH oxidation of microsomes. NADPH-stimulated respiration of the standard 2-ml microsomal suspension was monitored polarographically in a closed temperature-regulated (37°) chamber with a platinum electrode. The incubation mixture was the same as for ethanol oxidation except that sodium azide (5 mM) was added to inhibit catalase. Rates of NADPH oxidation were determined fluorometrically as described by Estabrook (15).

Chemicals. All cofactors and enzymes were purchased from either Boehringer or Sigma. All other chemicals were reagent grade products from standard commercial sources.

RESULTS

Effect of chronic ethanol treatment on animal growth. Both control and ethanol-treated animals exhibited comparable, positive weight gains with the liquid diet. However, the average weights of the ethanol-treated rats were slightly less than controls (Table 1). On the other hand, Porta and Gomez-Dumm (16) have demonstrated that biochemical and morphological changes need not necessarily be associated with an anabolic growth phase. Liver weights and the amount of microsomal protein isolated per gram of liver were essentially unchanged by the ethanol treatment (Table 1).

Effect of chronic ethanol treatment on microsomal ethanol oxidation. As has been reported by other workers (2, 17), prior treatment of animals with ethanol increases the ability of microsomes to oxidize ethanol in the presence of NADPH. In these experiments the oxidation occurred at a rate 27% greater in microsomes from treated than from control animals (Table 1), an increment which is statistically significantly different from the controls.

Effect of chronic ethanol treatment on H_2O_2 production. In this series of experiments, the decrease in scopoletin fluorescence in the presence of horseradish peroxidase was used as an assay system for the formation of H_2O_2 microsomes.

A typical experiment with the scopoletin method is shown in Fig. 1. The addition of an NADPH-generating system to microsomes from control animals produced a steady production of H_2O_2 (Fig. 1). On the other hand, the decrease in scopoletin fluorescence, i.e., the rate of H_2O_2 production, was much greater (62%) in microsomes from ethanol-treated animals (Fig. 1). A statistically significant increase in NADPH-dependent microsomal H_2O_2 production was observed (47%, $p < 0.005$) after treatment with ethanol (Table 1). Similar data (not shown) were obtained with the cytochrome *c* peroxidase method as an independent assay for H_2O_2. Thus enhanced NADPH-dependent H_2O_2 production in microsomes from ethanol-treated animals was apparent with two independent assay techniques.

Cytochrome P-450 contents, oxygen uptake, and NADPH oxidation. In microsomes from

TABLE 1

Effect of chronic ethanol treatment on microsomal NADPH and ethanol oxidation, oxygen uptake, and hydrogen peroxide production

Microsomes from control and ethanol-treated rats were compared. Data represent means ± standard errors except for the H_2O_2 data, which are means ± standard deviations. Measurements are described under MATERIALS AND METHODS.

Treatment	*n*	Rat weight	Liver wet weight	Microsomal protein isolated	Cytochrome P-450	NADPH oxidation	Ethanol oxidation	Oxygen uptake	H_2O_2 production
		g	*g*	*mg/g*	*nmoles/g*	*nmoles/min/mg*			*arbitrary units*
Control	16	139 ± 4	6.6 ± 0.4	20.5 ± 2.0	0.7 ± 0.1	12.8 ± 1.7	6.2 ± 0.5	18.8 ± 2.0	100 ± 4
Ethanol	16	124 ± 7	6.6 ± 0.3	19.3 ± 1.6	1.3 ± 0.2	16.5 ± 2.4	8.3 ± 0.4	26.6 ± 3.2	145 ± 7

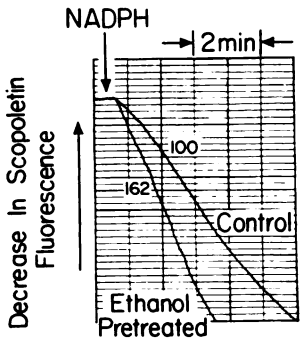

FIG. 1. *Effect of ethanol treatment on NADPH-dependent microsomal hydrogen peroxide formation*

Microsomes were prepared from animals receiving a control or an ethanol-containing diet for 21–28 days. Scopoletin (1 μM) was added to a suspension of microsomes (control, 2.03 mg/ml; ethanol-treated, 2.01 mg/ml) incubated in 80 mM potassium phosphate buffer, pH 7.4, containing 20 mM nicotinamide, 10 mM $MgCl_2$, and horseradish peroxidase (5 units/ml). The fluorescence intensity excited at 366 nm was recorded at 450 nm. An NADPH-generating system (0.4 mM $NADP^+$, 30 mM DL-isocitrate, and 200 milliunits/ml of isocitrate dehydrogenase) was added as indicated by the arrow.

ethanol-treated animals a significant increase in the content of cytochrome P-450 was observed, in confirmation of the finding of Mezey (18) (Table 1). Furthermore, significant increases in NADPH oxidation rates as well as in rates of oxygen uptake in the absence of added substrates were also seen in ethanol-treated animals (Table 1). However, interpretation of the stoichiometry of O_2:H_2O_2:NADPH was complicated by the presence of catalase as a microsomal contaminant.

DISCUSSION

Ethanol oxidation in vivo vs. in vitro. While it is generally agreed that the predominant pathway for ethanol oxidation in liver involves the alcohol dehydrogenase system, precise quantitation has remained difficult. For example, inhibition of ethanol oxidation of 60–80% has been observed *in vivo* and in perfused rat livers with specific inhibitors of alcohol dehydrogenase, pyrazole and 4-methylpyrazole[1] (19). Furthermore, about 80% inhibition of ethanol utilization by the perfused liver was observed in the presence of antimycin A, an inhibitor of the respiratory chain that prevents reoxidation of mitochondrial NADH (19). Inhibition of the respiratory chain would limit the supply of the oxidized cofactor (NAD^+) for the alcohol dehydrogenase reaction. Thus a reasonable estimate for the contribution of the alcohol dehydrogenase system to over-all ethanol oxidation in liver would be at least 80% of the total.

On the other hand, the nature of the alcohol dehydrogenase-independent pathways(s) is unknown. In intact cells there appear to be two possibilities to explain this residual ethanol oxidizing activity: (a) the oxidation of ethanol by the catalase–hydrogen peroxide complex and (b) the microsomal ethanol-oxidizing system. In addition, activity involving alcohol dehydrogenase with extramitochondrial reoxidation of NADH may occur in the experiments with antimycin A. Because the microsomal system requires NADPH instead of NAD^+, it appears that only the first two of the above possibilities could be operative in the isolated microsomal subfraction.

Role of NADPH-dependent hydrogen peroxide formation and catalase in microsomal ethanol oxidation. The observation by Orme-Johnson and Ziegler (20) that the microsomal fraction of liver can oxidize ethanol in the presence of NADPH and oxygen has generated considerable interest and controversy in the past several years. At the time of the original work the role of catalase in this reaction was minimized because added H_2O_2 did not support the oxidation. However, it was later demonstrated that added H_2O_2 could not be recovered from microsomes (21), presumably because of its rapid conversion into oxygen and water by the catalase present in the suspension. Conversely, a possible role for catalase in microsomal ethanol oxidation was suggested by the observation that ethanol oxidation by the microsomal subfraction was supported by a continuous supply of H_2O_2 (1).

Lieber and DeCarli (2) have demonstrated several similarities between the microsomal ethanol-oxidizing system and classical mixed

[1] H. G. Ley, R. G. Thurman, P. Zimmer, and R. Scholz, unpublished observations.

function oxidases: (a) requirements for NADPH and oxygen and (b) partial inhibition by carbon monoxide. They have implied from their work that the microsomal system is a unique ethanol-oxidizing system and have demonstrated that it is inducible by chronic ethanol treatment. They have also indicated that this system is different from catalase, based on differential sensitivity to catalase inhibitors depending upon whether H_2O_2 is generated from NADPH or an artificial system such as xanthine oxidase plus hypoxanthine. However, failure to control the rate of generation of H_2O_2 has led Oshino, Oshino, and Chance (22) to criticize this interpretation.

The hypothesis that a unique microsomal ethanol-oxidizing system exists has been criticized by a large number of laboratories, and evidence has accumulated strongly implicating the H_2O_2-catalase system in microsomal ethanol oxidation. For example, the demonstration that the microsomal ethanol-oxidizing system can be supported by an H_2O_2-generating system (glucose plus glucose oxidase; hypoxanthine plus xanthine oxidase) and can be inhibited by catalase inhibitors clearly implicates the Keilin-Hartree coupled oxidation in the peroxidation of ethanol in the microsomal subfraction (1–4). Furthermore, it has been demonstrated recently that microsomes produce H_2O_2 in reactions which require oxygen and NADPH, and which can be partially inhibited by carbon monoxide (4). Also, H_2O_2 production and ethanol oxidation have the same oxygen profiles (K_m = 50 μM; [O_2] at V_{max} = 200 μM). These characteristics are identical for microsomal H_2O_2 production, implicating H_2O_2-catalase in the microsomal ethanol-oxidizing system. Furthermore, substrates for catalase (formate, ascorbate) and H_2O_2-utilizing systems (horseradish peroxidase plus hydrogen donors) inhibit the system; these observations led Thurman, Ley, and Scholz to conclude that since the characterization upon which a unique microsomal ethanol-oxidizing system hypothesis was based is compatible with a microsomal H_2O_2-catalase mechanism, it is superfluous to postulate a new microsomal ethanol-oxidizing system in addition to the peroxidative reaction of catalase (see Fig. 2).

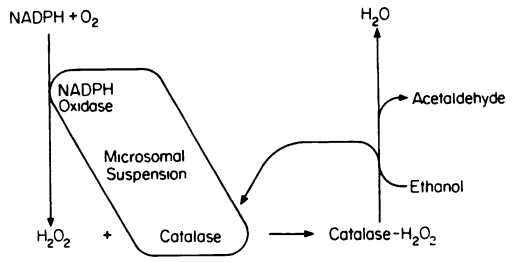

FIG. 2. *Scheme depicting pathway for ethanol oxidation in microsomes*

Recently a number of workers have attempted to resolve these controversies by examining whether or not an active, reconstituted, catalase-free mixed-function oxidation system is capable of oxidizing ethanol. Teschke *et al.* (23) have reported that some ethanol is oxidized in fractions free of catalase. However, these experiments have not been confirmed by others (24). On the contrary, work from Coon's laboratory with four different types of purified P-450 demonstrated the inability of the reconstituted mixed-function oxidation system to oxidize ethanol as judged by the effect of this compound on NADPH disappearance.[2] Also, Mezey *et al.* (5) have reported ethanol oxidation by a microsomal fraction rich in cytochrome P-450. However, their preparation admittedly contained catalase, and azide sensitivity was demonstrated, characteristics which are consistent with a catalase-H_2O_2 mechanism for the oxidation.

Adaptive increases in microsomal H_2O_2 production due to chronic ethanol treatment. It has been demonstrated that chronic treatment of animals with ethanol produced pronounced morphological changes in the ultrastructure of the liver (25). Among these changes are the appearance of "bizarre" mitochondria, the accumulation of fat droplets, and a proliferation of the membranes of the endoplasmic reticulum. In addition, altered biochemical parameters were also observed. Diminished rates of pyruvate oxidation (26) and enhanced succinoxidase (27) have been observed following chronic ethanol treatment in studies on isolated mitochondria and liver slices, respectively. In addi-

[2] J. Vermilion, R. M. Kaschnitz, and M. J. Coon, unpublished observations.

tion, a large number of changes in enzyme activities of the smooth endoplasmic reticulum have been reported following chronic treatment with ethanol. An increase in cytochrome P-450 content, an increase in NADPH-dependent endogenous respiration (Table 1), and an increase in the rate of NADPH oxidation all accompany chronic ethanol treatment (Table 1).

Furthermore, the adaptive increase in microsomal H_2O_2 production reported here (Fig. 1 and Table 1) and its parallelism with increases in acetaldehyde formation from ethanol (Table 1) are consistent with the postulate that microsomal ethanol oxidation is due to the catalase-H_2O_2 system (Fig. 2), since production of H_2O_2 is rate-limiting for the catalase reaction in the cell (4).

REFERENCES

1. M. K. Roach, W. N. Reese, Jr., and P. J. Creaven, *Biochem. Biophys. Res. Commun.* **36,** 596–602 (1969).
2. C. S. Lieber and L. M. DeCarli, *J. Biol. Chem.* **245,** 2505–2512 (1970).
3. K. J. Isselbacher and E. A. Carter, *Biochem. Biophys. Res. Commun.* **39,** 530–537 (1970).
4. R. G. Thurman, H. G. Ley, and R. Scholz, *Eur. J. Biochem.* **25,** 420–430 (1972).
5. E. Mezey, I. I. Potter, and W. D. Ree, *J. Biol. Chem.* **248,** 1183–1187 (1973).
6. C. S. Lieber and L. M. DeCarli, *Science* **170,** 78–80 (1970).
7. C. S. Lieber, *in* "Metabolic Changes Induced by Ethanol" (G. A. Martini and C. Bode, eds.) pp. 85–92. Springer, New York, 1970.
8. L. M. DeCarli and C. S. Lieber, *J. Nutr.* **91,** 331–336 (1967).
9. A. Hildebrandt, H. Remmer, and R. W. Estabrook, *Biochem. Biophys. Res. Commun.* **30,** 607–612 (1968).
10. A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.* **177,** 751–766 (1949).
11. N. K. Gupta and W. G. Robinson, *Biochim. Biophys. Acta* **118,** 431–434 (1966).
12. A. Boveris, N. Oshino, and B. Chance, *Biochem. J.* **128,** 617–630 (1972).
13. W. A. Andreae, *Nature* **175,** 859–860 (1955).
14. T. Omura and R. Sato, *J. Biol. Chem.* **239,** 2370–2378 (1964).
15. R. W. Estabrook, *Anal. Biochem.* **4,** 231–245 (1962).
16. E. A. Porta and C. L. A. Gomez-Dumm, *Lab. Invest.* **18,** 352–364 (1968).
17. K. J. Isselbacher and E. A. Carter, *Drug Metab. Dispos.* In press.
18. E. Mezey, *Biochem. Pharmacol.* **21,** 137–142 (1972).
19. R. G. Thurman, *Fed. Proc.* **32,** 1429 (1973).
20. W. H. Orme-Johnson and D. M. Ziegler, *Biochem. Biophys. Res. Commun.* **21,** 78–82 (1965).
21. V. Ullrich, *in* "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, eds.) p. 222. Academic Press, New York, 1969.
22. N. Oshino, R. Oshino, and B. Chance, *Biochem. J.* **131,** 555–567 (1973).
23. R. Teschke, Y. Hasumura, J. G. Joly, H. Ishii, and C. S. Lieber, *Biochem. Biophys. Res. Commun.* **49,** 1187–1193 (1972).
24. R. G. Thurman and R. Scholz, *Drug Metab. Dispos.* **1,** 441–448 (1973).
25. E. Rubin, F. Hutterer, and C. S. Lieber. *Science* **159,** 1469–1470 (1968).
26. K.-H. Kiessling and K. Tilander, *Exp. Cell Res.* **30,** 476–480 (1963).
27. M. Videla and Y. Israel, *Biochem. J.* **118,** 275–281 (1970).