Biochemical Pharmacology, Vol. 35, No. 24, pp. 4590–4592, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Characterization of hamster liver nicotine metabolism—II. Differential effects of ethanol or phenobarbital pretreatment on microsomal N and C oxidation

(*Received* 27 *February* 1986; *accepted* 26 *June* 1986)

Numerous studies have established that in men heavy consumption of alcoholic beverages is accompanied frequently by heavy cigarette smoking [1–3]. In addition to the negative health consequences of excessive exposure to either tobacco or alcohol, both prospective and retrospective epidemiological studies have established that cancers of the head and neck are increased significantly in men who are heavy users of both tobacco and alcohol [4–7]. Enhanced rates of metabolic activation of tobacco-associated carcinogens have been suggested as one mechanism which could account for the increased risk for cancer in smokers who are also drinkers [8]. Several studies have shown that both the microsomal metabolism and the carcinogenicity of certain chemical carcinogens can be increased in experimental animals by chronic ethanol consumption [9–12]. It has been suggested that increased tobacco usage in heavy drinkers is the result of increased rates of nicotine metabolism, resulting in an increased need to smoke in order to maintain satisfying blood nicotine levels [13]. Despite evidence for pronounced effects of ethanol consumption on drug metabolism in experimental animals and man, no studies examining the effect of ethanol pretreatment on microsomal nicotine metabolism have been reported. It is the purpose of this communication to compare the effect of pretreatment of hamsters with ethanol or phenobarbital on the relative rates of microsomal nicotine N and C oxidation. Administration of ethanol as 18.5% ethanol–water solution for 28 days increased the rate of nicotine *N'*-oxidase activity but had no effect on the rate of nicotine 5'-hydroxylase activity. In contrast, phenobarbital pretreatment had no effect on the rate of nicotine *N'*-oxidase activity but markedly increased the rate of nicotine 5'-hydroxylase activity.

Materials and methods

Chemicals. Potassium cyanide, nicotinamide adenine dinucleotide phosphate (monosodium salt), nicotinamide, Tricine (*N*-tris-[hydroxymethyl]-methyl glycine), Tris (tris-[hydroxymethyl]-amino-methane), glucose-6-phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Type XII) were obtained from the Sigma Chemical Co., St. Louis, MO; (*S*)-(-)nicotine and sucrose (special enzyme grade) were from Schwarz/Mann, Orangeburg, NY.

Animals and treatment. Six-week-old male LVG Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Charles River Breeding Laboratories, Lakeview Colony, Newfield, NJ. Animals were housed two per cage in regulation stainless steel cages and given free access to NIH-07 lab chow (Ziegler Brothers, Gardners, PA) and tap water. At 8 weeks of age, animals were randomized by weight into three groups of six animals each. At 9 weeks of age, one group was given free access to 18.5% ethanol–water in place of drinking water, as described previously [14, 15], while the other two groups were continued on tap water. At 12 weeks of age, a second group of six animals was given daily intraperitoneal injections of phenobarbital (80 mg/kg body wt/day in saline) for 4 consecutive days as described previously [16]. All animals were killed by decapitation at 13 weeks of age.

Subcellular fractionation and biochemical analysis. Livers were rapidly removed, weighed, minced, rinsed three times with 10 vol. of ice-cold SET (0.3 M sucrose, 0.5 mM EDTA, and 5 mM Tricine, pH 7.4), and suspended in 9 vol. of ice-cold SET. Homogenization and subcellular fractionation were performed as described previously [16]. Cytochrome P-450 content was determined according to the method of McLean and Day [17] and cytochrome b_5 content as described by Omura and Sato [18]. Protein was determined by the method of Lowry *et al.* [19]. Statistical significance was evaluated using Student's two-tailed *t*-test.

In vitro *nicotine metabolism.* The standard assay conditions for determination of the rates of nicotine N and C oxidation consisted of: 2 μmoles nicotinamide, 1 μmole $NADP^+$, 5 μmoles glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase, 6 μmoles $MgCl_2$, 30 μmoles nicotine, 1 μmole potassium cyanide, and 40 μmoles Tricine, pH 7.4, in a final volume of 1.0 ml in a 25-ml Erlenmeyer flask. Reactions were initiated by the addition of microsomes (0.6 to 1.2 mg protein). Following incubation for 30 min at 37° in a Dubnoff metabolic shaking incubator, reactions were quenched by the addition of 1.0 ml of ice-cold acetone. After removal of protein by centrifugation, a 1.0-ml aliquot of the supernatant fraction was extracted with 5.0 ml chloroform. Five milliliters of the chloroform extract was evaporated under a stream of nitrogen, and the residue was redissolved in 0.10 ml methanol and saved for analysis of 5'-cyanonicotone. The extracted aqueous fraction was saved from determination of nicotine *N'*-oxide.

HPLC separations. Analyses were performed using a Varian series 5000 high pressure liquid chromatograph equipped with a Varian UV 100 variable wavelength detector and a Rheodyne injector and a Varian 4 mm × 30 cm Micropak MCH-10 column, as described previously [20]. Reservoir A was water adjusted to pH 9.5–9.7 by the addition of 5 drops of concentrated ammonium hydroxide per liter. Reservoir B was 100% methanol. Flow rates were 2 ml/min, and effluents were monitored at 254 nm. Specific eluting conditions are included in the figure legend.

Results and discussion

Cotinine, the major C-oxidation metabolite of nicotine, is not a primary microsomal metabolite, but is formed by the oxidation of the initial microsomal product nicotine Δ1',5'-iminium ion by cytosolic aldehyde oxidase [21]. Murphy [22] has shown that nicotine oxidation by this pathway can be estimated by taking advantage of the fact that the Δ1',5'-iminium ion can be trapped with potassium cyanide as the 5'-cyano adduct of nicotine.

Because N and C oxidation of nicotine appear to involve distinct microsomal enzymatic pathways [22–24], it was of interest to examine the effect of pretreatment with either ethanol or phenobarbital on the microsomal metabolism of nicotine. The effects of variations in nicotine concentration on the rates of 5'-cyanonicotine and nicotine *N'*-oxide formation in microsomes isolated from control, ethanol-pretreated and phenobarbital-pretreated hamsters are presented in Fig. 1. Phenobarbital markedly increased the rate of 5'-cyanonicotine formation, whereas the rate for ethanol-pretreated microsomes was similar to the control rate (Fig. 1A). In contrast, the rate of nicotine *N'*-oxide formation remained unchanged in phenobarbital-pretreated microsomes, whereas a dramatic increase in the rate was observed when microsomes from ethanol-pretreated animals were assayed (Fig. 1B). Maximal rates of formation of both metabolites were achieved at substrate concentrations greater than 30 mM in all three microsomal

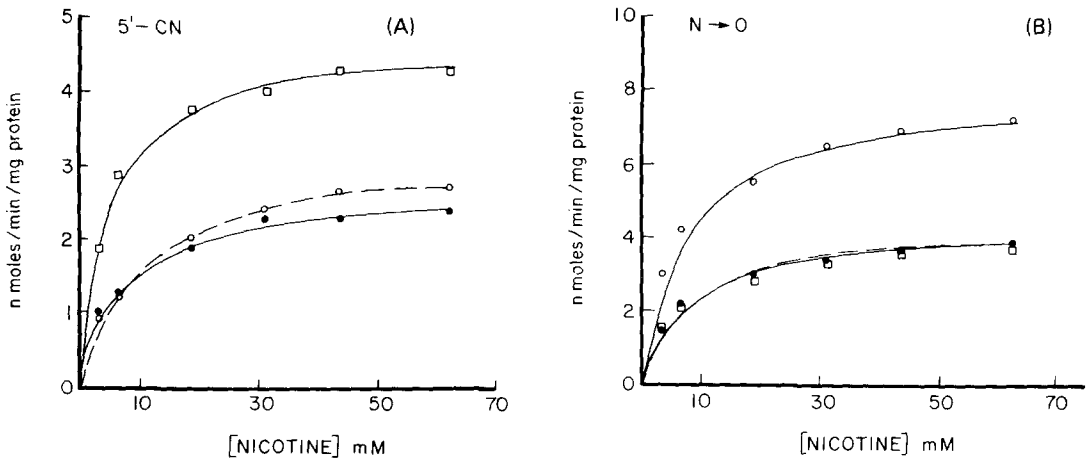

Fig. 1. (A) Effect of variations in nicotine concentration on the rate of 5′-cyanonicotine formation. Twenty-microliter aliquots of the chloroform extracts were separated by gradient elution. Gradient conditions were: zero to 10 min, 20% B; 10 to 20 min, 20–35% B; 20 to 30 min, 35–70% B; 30 to 33 min, 70–80% B. Metabolites and standards eluted in the following order: cotinine (19.2 min), methylcyanonicotine (27.2 min), nornicotine (29.2 min), 5′-cyanonicotine (30.5 min), and nicotine (35.0 min). Key: (●) control, (○) ethanol, and (□) phenobarbital pretreatment and microsomes. (B) Effect of variations in nicotine concentration on the rate of nicotine *N*′-oxide formation. Thirty-microliter aliquots of the aqueous extracts were separated using isocratic elution at 70% methanol, 30% water. Nicotine *N*′-oxide eluted at 5.3 min and nicotine at 9.7 min. Key: (●) control, (○) ethanol, and (□) phenobarbital pretreatment and microsomes.

preparations. Lineweaver–Burk analysis of the data in Fig. 1 indicated that inducer pretreatment altered maximal velocities. The apparent K_m for nicotine (5–7 mM) was unaffected (data not shown).

The effects of ethanol and phenobarbital pretreatment on microsomal cytochromes and the rates of N and C oxidation of nicotine are presented in Table 1. Our previous studies have demonstrated that administration of this level of ethanol in the drinking water causes no change in total microsomal P-450 content but does induce both aniline hydroxylase (high-affinity form) and *p*-nitroanisole *O*-demethylase activity [15, 16]. We also observed previously the decrease in the specific content of cytochrome b_5 following ethanol administration and showed that this decrease in cytochrome b_5 is accompanied by lowered rates of benzo[*a*]pyrene metabolism [25] and ethoxyresorufin *O*-deethylase activity [15]. Ethanol pretreatment had no effect on the rate of 5′-hydroxylation of nicotine but *N*′-oxidation was significantly increased almost 2-fold. The participation of cytochrome b_5 in the N or C oxidation of nicotine would appear unlikely since neither rate was decreased by ethanol pretreatment.

The increases in both total cytochrome P-450 and b_5 as a result of phenobarbital pretreatment (Table 1) are similar to those reported previously [16, 26]. Our previous studies showed that phenobarbital pretreatment of hamsters results in small increases in both the low- and high-affinity form of aniline hydroxylase [16], the 5′-hydroxylation of *N*′-nitrosonornicotine, arylhydrocarbon hydroxylase activity [26], and both the α-hydroxylation and the metabolic activation of *N*-nitrosopyrrolidine [27]. The rate of 5′-hydroxylation of nicotine was increased similarly whereas no change in the rate of *N*′-oxidation was observed. The studies of Nakayama *et al.* [28–30] showed that phenobarbital pretreatment of rats or guinea pigs results in enhanced rates of nicotine oxidase activity. However, because this assay is based on measuring the rate of nicotine disappearance, the specific pathway of nicotine metabolism being induced could not be determined.

The effect of either ethanol or phenobarbital pretreatment on the pharmacokinetics of nicotine by hamsters has not yet been investigated. The fact that ethanol consumption by hamsters selectively enhanced the rate of nicotine *N*′-oxide formation whereas phenobarbital pre-

Table 1. Differential effects of ethanol and phenobarbital pretreatment on hamster microsomal nicotine metabolism

Pretreatment	Cytochrome P-450 (nmoles/mg)	Cytochrome b_5 (nmoles/mg)	Nicotine 5′-hydroxylase (nmoles 5′-cyanonicotine formed/min/mg)	Nicotine *N*′-oxidase (nmoles nicotine *N*′-oxide formed/min/mg)
Control	1.03 ± 0.07 (6)	0.61 ± 0.05 (6)	2.84 ± 0.44 (6)	4.16 ± 0.52 (6)
Ethanol	1.02 ± 0.19 (6)	0.36 ± 0.05* (6)	2.43 ± 0.52 (6)	8.02 ± 1.08* (6)
Phenobarbital	1.66 ± 0.11* (5)	0.67 ± 0.02† (5)	5.67 ± 0.49* (5)	3.95 ± 0.48 (5)

Rates of nicotine N and C oxidation were measured as described under Materials and Methods. The nicotine concentration was 30 mM. All values are means ± S.D. Numbers in parentheses are the number of separate liver microsomal preparations.

* $P < 0.01$ from control.

† $P < 0.05$ from control.

treatment selectively enhanced the rate of 5'-cyanonicotine formation shows quite clearly that there is great potential for numerous drugs including ethanol to modify nicotine metabolism by selectively altering the flux of nicotine through its various metabolic pathways.

Acknowledgements—This investigation was supported by PHS Grant CA 32126 awarded by the National Cancer Institute. The authors wish to thank Dr. Hasan Mukhtar for his many helpful discussions and Maria Birkel for preparation of the manuscript.

Department of Environmental Health Sciences
Case Western Reserve University
School of Medicine
Cleveland, OH 44106, U.S.A.

G. DAVID MCCOY*
GEORGE J. DEMARCO

REFERENCES

1. R. G. Walton, *Am. J. Psychiat.* **128**, 1455 (1972).
2. R. M. Maletzky and J. Klotter, *Am. J. Psychiat.* **131**, 445 (1974).
3. R. R. Griffiths, G. E. Bigelow and I. Liebson, *J. expl Analys. Behav.* **25**, 279 (1976).
4. E. L. Wynder, I. J. Bross and E. A. Day, *Cancer* **9**, 86 (1956).
5. E. Rothman and A. Z. Keller, *J. chronic Dis.* **25**, 711 (1972).
6. S. Graham, *J. natn. Cancer Inst.* **59**, 1611 (1977).
7. W. Schmidt and R. E. Popham, *Cancer* **47**, 1031 (1981).
8. G. D. McCoy and E. L. Wynder, *Cancer Res.* **39**, 2844 (1979).
9. M. J. Radike, K. L. Stemmer, P. G. Brown, E. Larson and E. Bingham, *Environ. Hlth Perspect.* **21**, 153 (1977).
10. G. D. McCoy, S. S. Hecht, S. Katayama and E. L. Wynder, *Cancer Res.* **41**, 2849 (1981).
11. L. Gircíute, M. Castegnaro and J-C. Bereziat, in *N-Nitroso Compounds: Occurrence and Biological Effects* (Eds. H. Bartsch, I. K. O'Neill, M. Castegnaro and M. Okada), pp. 643–8. International Agency for Research on Cancer, IARC Scientific Publications No. 41, Lyon, France (1982).
12. A. Castonguay, A. Rivenson, N. Trushin, J. Reinhart, S. Spathopoulos, C. J. Weiss, B. Reiss and S. S. Hecht, *Cancer Res.* **44**, 2285 (1984).
13. J. Adir, W. Wildfeuer and R. P. Miller, *J. Pharmac. expl Ther.* **212**, 274 (1980).
14. G. D. McCoy, A. D. Haisley, P. Powchik and P. C. Tambone, *J. Stud. Alcohol* **42**, 508 (1981).
15. G. D. McCoy, G. J. DeMarco and J. A. Biaglow, *Biochem. Pharmac.* **9**, 4263 (1985).
16. G. D. McCoy, *Biochem. Pharmac.* **29**, 685 (1980).
17. A. E. M. McLean and P. A. Day, *Biochem. Pharmac.* **23**, 1183 (1974).
18. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. G. D. McCoy, P. C. Howard and G. J. DeMarco, *Biochem. Pharmac.* **35**, 2767 (1986).
21. H. B. Hucker, J. R. Gillette and B. B. Brodie, *J. Pharmac. expl Ther.* **129**, 94 (1960).
22. P. J. Murphy, *J. biol. Chem.* **248**, 2796 (1973).
23. J. Booth and E. Boyland, *Biochem. Pharmac.* **20**, 407 (1971).
24. P. Jenner, J. W. Gorrod and A. H. Beckett, *Xenobiotica* **3**, 563 (1973).
25. G. J. DeMarco and G. D. McCoy, *Biochem. biophys. Res. Commun.* **128**, 621 (1985).
26. G. D. McCoy, C-H. B. Chen and S. S. Hecht, *Drug Metab. Dispos.* **9**, 168 (1981).
27. G. D. McCoy, S. S. Hecht and E. C. McCoy, *Environ. Mutagen.* **4**, 221 (1982).
28. H. Nakayama, T. Nakashima and Y. Kurogochi, *Biochim. biophys. Acta* **715**, 254 (1982).
29. H. Nakayama, T. Nakashima and Y. Kurogochi, *Biochim. biophys. Res. Commun.* **108**, 200 (1982).
30. H. Nakayama, T. Nakashima and Y. Kurogochi, *Biochem. Pharmac.* **34**, 2281 (1985).

* Author to whom all correspondence should be addressed.

Biochemical Pharmacology, Vol. 35, No. 24, pp. 4592–4594, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00

Role of hepatic glutathione and glucocorticoids in the regulation of hepatic cholesterol 7α-hydroxylase

(*Received* 11 *April* 1986; *accepted* 7 *July* 1986)

Hepatic microsomal cholesterol 7α-hydroxylase (CH-7A, EC 1.14.13.7) is the rate-limiting enzyme of bile acid biosynthesis from cholesterol [1]. CH-7A has an important role in cholesterol homeostasis since the conversion of cholesterol to bile acids is quantitatively a very important pathway of removal of cholesterol from the body [2].

Recently, Hassan *et al.* [3] provided evidence, for the first time, for a role of hepatic reduced glutathione (GSH) content in the regulation of CH-7A activity. They showed that acute changes in hepatic GSH content *in vivo* significantly affect CH-7A activity assayed *in vitro* [3]. Depletion of hepatic GSH (using diethylmaleate [DEM]) significantly reduces CH-7A activity, whereas partial repletion of GSH (using L-cysteine), in previously GSH-depleted animals, partially restores CH-7A activity, although it is still significantly less than that in GSH-replete controls [3]. Based on these observations, Hassan *et al.* [3] suggested that physiological variations in hepatic GSH content may regulate CH-7A activity. Since both hepatic GSH content and CH-7A activity are known to be sensitive to the feeding status of the animal [4, 5], then it may be hypothesized that there is a temporal link between feeding and subsequent changes in hepatic GSH content and CH-7A activity.

The objective of this study was to show that there is a direct relation between the feeding-related change in hepatic GSH content and hepatic CH-7A activity. Additionally, since glucocorticoids are known to affect CH-7A activity [6], the effect of adrenalectomy (ADX) on CH-7A activity was studied to determine if there were any glucocorticoid/GSH interactions.

Methods

Animals and treatment. Male, Sprague–Dawley rats weighing between 40 and 45 g were obtained from the Holtzman Rat Co. (Madison, WI) and adapted to a 10-hr light cycle (8:00 a.m. to 8:00 p.m.). Food was only available for 2 hr between 8:00 and 10:00 a.m., whereas water was