Effect of Ethanol on CHCl₃ Metabolism in Hepatic Microsomes from Osborne-Mendel Rats

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The treatment of Osborne-Mendel rats with ethanol in drinking water for 2 weeks resulted in a 3-fold increase of hepatic microsomal hydroxylation of both p-nitrophenol and aniline, two substrates considered highly selective for P4502E1. No other forms of P450 seemed to be affected. These results, confirmed by the immunoblot analysis of microsomal protein, showed an induction of P4502E1. The levels of total covalent binding to microsomal phospholipid due to [¹⁴C]CHCl₃ reactive intermediates in ethanol-pretreated microsomes were identical to those measured in microsomes from untreated rats at any pO₂. The distribution of radioactivity obtained after transmethylation of the adducts of [¹⁴C]CHCl₃ intermediates with microsomal phospholipids (PL) indicated that binding to fatty acyl chains (due to -CHCl₂ radicals) increased with decreasing pO₂. On the contrary, the binding to polar heads due to phosgene decreased. The ethanol treatment did not affect binding to either PL moieties. These results indicated that, in our experimental conditions, the in vitro production of both oxidative and reductive intermediates of CHCl₃ in the liver of Osborne-Mendel rats were not influenced by ethanol consumption.—Environ Health Perspect 102(Suppl 9):25-30 (1994)

Key words: CHCl₃ activation, ethanol induction, P4502E1, liver microsomes, Osborne-Mendel rats, covalent binding

Introduction

The potentiation of CHCl₃-induced hepatotoxicity in rodents by ethanol (1) and other aliphatic alcohols (2,3), ketones and ketogenic compounds (4-8) is well known.

Various mechanisms have been proposed in order to explain this effect, such as increased absorption in the intestine (9), depletion of liver GSH (10,11), inhibition of hepatocellular regeneration and hepatolobular restoration (3,7), and hepatic hypermetabolism (12). However, the leading hypothesis refers to cytochrome P450 induction, responsible for an increase in CHCl₃ metabolism (6,13-15). The potentiating effects among alcohols and ketones differ significantly; this finding has been related to their qualitatively and quantitatively different capacities to induce various P450 isoenzymes (16). The involvement of P4502B1/2 has been evidenced (14) but much attention was drawn by P4502E1 (15) since it is the main ethanol- and acetone-inducible form (17) able to catalyze CHCl₃ biotransformation (13).

Until recently, studies on the association of the enhancement of CHCl₃-induced hepatotoxicity with its metabolism were related only to the detection of the CHCl₃ oxidation reactive intermediate, phosgene (6,14). No data are reported on the role of the reductive pathway, through which CHCl₃ is biotransformed to radical species (CHCl₂) (18,19), able to bind covalently to cellular structures (20,21).

Due to the social relevance of ethanol, to which most of human population can be exposed, we decided to study its interaction with CHCl₃, although this alcohol is not the most effective potentiator of CHCl₃ hepatotoxicity. We investigated the effect of ethanol pretreatment on the P450-dependent drug-metabolizing system in Osborne-Mendel hepatic microsomes. Then we investigated the effect produced by the same pretreatment on both oxidation and reduction pathways of CHCl₃ metabolism, using a CHCl₃ concentration (5 mM) at which both metabolic pathways are expressed (21).

Materials and Methods

Chemicals

(¹⁴C)-Chloroform (3.6 mCi/mmmole, radiochemical purity 99%) was obtained from New England Nuclear (Boston, MA). Unlabeled chloroform (IR purity) was from Merck (Darmstadt, Germany). Liquid scintillation cocktails Aqualuma and Lipoluma were purchased from Lumar Systems A.G. (Basel, Switzerland). Resorufin was obtained from Fluka (Buchs, Switzerland). 7-Ethoxyconumarin was from EGA-Chemie (Steinheim, Germany). Benzphetamine was supplied by Upjohn Co. (Kalamazoo, Michigan). Ethoxyresorufin and pentoxyresorufin were synthesized from resorufin by ethylation with ethyl iodide and by pentylation with pentyl iodide, respectively (22).

Enzymes and coenzymes were obtained from Boehringer GmbH (Mannheim, Germany). Nitrocellulose filters (0.45 μm), erythromycin, testosterone (T), 4-androsten-3,17-dione (17 OT), 16β-hydroxytestosterone (16β-OH), and corticosterone were supplied by Sigma Chemicals (St. Louis, MO). 2α-,2β-, 6α-, 6β-, 7α- and 16α-hydroxytestosterone (16α-OH) were obtained from the Steroids Reference Collection (DN Kirk, Department of Chemistry, Queen Mary College, London, England). Rabbit anti-rat P450
2E1 polyclonal antibodies were purchased from Oxygene (Dallas, Texas).

All other analytical grade chemicals were obtained from common commercial sources.

**Animals**

Male Osborne-Mendel rats (180–200 g) were from Zentralinstitut für Versuchstierzucht (Hannover, Germany). They were maintained on a 12-hr light cycle and provided food and water *ad libitum* for 1 week and then administered 15% (vol/vol) ethanol in drinking water for 2 weeks. Liver microsomal preparation were obtained as previously described (23).

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### Table 1. Effect of ethanol administration on monooxygenase activities in hepatic microsomes from Osborne-Mendel rats. 9

| Enzymatic activity | Control microsomes | Ethanol-treated microsomes |
|--------------------|--------------------|---------------------------|
| P450<sup>a</sup>   | 0.6 ± 0.08         | 0.54 ± 0.11               |
| bs<sup>a</sup>     | 0.14 ± 0.01        | 0.16 ± 0.03               |
| APND<sup>a</sup>   | 5.3 ± 0.91         | 4.7 ± 0.6                 |
| BZND<sup>a</sup>   | 4.9 ± 0.46         | 5.7 ± 0.80                |
| ErND<sup>a</sup>   | 0.64 ± 0.08        | 0.50 ± 0.13               |
| AnOH<sup>a</sup>   | 0.49 ± 0.12        | 1.10 ± 0.21               |
| pNPH<sup>a</sup>   | 0.49 ± 0.006*      | 1.51 ± 0.008*             |
| ECOD<sup>a</sup>   | 0.55 ± 0.13        | 0.83 ± 0.15               |
| EROD<sup>a</sup>   | 0.11 ± 0.03        | 0.11 ± 0.02               |
| PROD<sup>a</sup>   | 7.6 ± 2.5          | 6.0 ± 1.0                 |

<sup>a</sup>nmole/mg proteins. <sup>b</sup>nmole/min/mg proteins. <sup>c</sup>pmole/min/mg proteins. Abbreviations: P450, cytochrome P450; bs, cytochrome b<sub>5</sub>; APND, aminopyrine N-demethylease; BZND, benzphetamine N-demethylease; ErND, erythromycine N-deethylase; AnOH, aniline hydroxylase; pNPH, p-nitrophenol hydroxylase; ECOD, 7-ethoxycoumarin-O-deethylase (ECOD) activity was assayed by the method of Aitio (29). Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-depentylation (PROD) activities were determined by measuring the formation of the corresponding hydroxy products (30).

**Biochemical Assays**

Microsomal protein content was determined by the method of Oyama and Eagle (24), using bovine serum albumin as a standard. Cytochrome P450 and cytochrome b<sub>5</sub> were measured by the method of Omura and Sato (25). The activities for the N-demethylation of aminopyrine (APND), benzphetamine (BZND), and erythromycine (ErND) were assayed by measuring the formation of formaldehyde (26). Aniline hydroxylase (AnOH) and p-nitrophenol hydroxylase (pNPH) were determined according to Ko et al. (27) and Reinke and Moyer (28). The 7-ethoxycoumarin-O-deethylase (ECOD) activity was assayed by the method of Aitio (29). Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-depentylation (PROD) activities were determined by measuring the formation of the corresponding hydroxy products (30).

Testosterone hydroxylase was assayed according to an HPLC method as described by Platt et al. (31).

Lipids were extracted according to Folch et al. (32).

**Gel Electrophoresis and Immunoblotting**

SDS-PAGE was carried out using the discontinuous system of Laemmli (33), using a 1.5-mm thick gel with 3 and 7.5% acrylamide in the stacking and separation gel. Proteins were transferred from the slab gel to the nitrocellulose filters, following the method of Towbin et al. (34). Immunodetection of P4502E1 was performed using rabbit polyclonal antibodies.

**In Vitro Activation of 14C-Chloroform**

The standard incubation mixture contained microsomal protein (2 mg/ml), G6P (2 mM), MgCl<sub>2</sub> (2 mM) G6P-dehydrogenase (1 U/ml), EDTA (1 mM), NADP (0.2 mM) and 14C-chloroform 5 mM in 50 mM Tris-Cl buffer, pH 7.4. When anoxic conditions were required, the incubation mixture also contained an oxygen scavenging enzyme system. Mixture to be incubated under hypoxic (about 1% PO<sub>2</sub>) or anoxic conditions were flushed with ultrapure N<sub>2</sub> for 20 min; a mixture of O<sub>2</sub> N<sub>2</sub> (5:95) was flushed for 20 min when incubations were to be carried out at 5% PO<sub>2</sub>- A detailed description of the procedure was reported previously (20).

**Covalent Binding of 14C-Chloroform Metabolites**

Covalent binding of 14C-label to microsomal lipid was measured after 20 min incubation according to the method of Uehleke (35), with minor modifications (20). The regioselective binding of 14C-chloroform metabolites to microsomal phospholipids (PL)-polar heads and/or fatty acyl chains was determined after the acid catalyzed transmethylation of PL-adducts, as described in detail by De Biasi et al. (36).

Briefly, the lipid extract was dissolved in 1 ml of anhidrous methanol: benzene: H<sub>2</sub>SO<sub>4</sub> (75:25:1), and vigorously shaken for 1 hr at 70°C. The reaction mixture was cooled in an ice bath, then 2 ml petroleum ether (bp 40°–70°) and 1 ml 0.26M K<sub>2</sub>HPO<sub>4</sub> were added. After 10 min vigorous shaking, the mixture was centrifuged (3000 rpm, 10 min). The lower aqueous

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### Table 2. Hydroxylation of testosterone by liver microsomes of control and ethanol-treated Osborne-Mendel rats. 9

| Metabolites | Control microsomes | Ethanol-treated microsomes |
|------------|--------------------|---------------------------|
| 6α-OH     | 0.0012 ± 0.007     | 0.018 ± 0.01              |
| 7α-OH     | 0.029 ± 0.01       | 0.025 ± 0.01              |
| 6β-OH     | 0.16 ± 0.04        | 0.12 ± 0.03               |
| 16α-OH    | 0.31 ± 0.11        | 0.34 ± 0.09               |
| 16β-OH    | 0.014 ± 0.006      | 0.010 ± 0.004             |
| 2α-OH     | 0.25 ± 0.05        | 0.22 ± 0.03               |
| 2β-OH     | 0.032 ± 0.008      | 0.024 ± 0.007             |
| 17-DT     | 0.33 ± 0.06        | 0.25 ± 0.05               |
| Total     | 1.13 ± 0.29        | 1.01 ± 0.23               |

<sup>a</sup>Results are expressed as nmol/min by mg protein. Values represent the mean ± standard deviation of four experiments performed with different microsomal preparations. Incubations were carried out at 37°C for 15 min with 1 mg/ml microsomal proteins.
layer, containing the hydrophyllic polar heads, was transferred into a plastic vial containing 17 ml Aquasol. The upper organic phase, containing the PL fatty acid methyl esters, was washed twice and then a 2-ml aliquot was transferred into a plastic vial containing 10 ml Lipoluma.

Calculations

Data obtained with control and ethanol treated microsomes were compared by means of the Student's t-test.

Results

Effects of Ethanol on Hepatic Drug Metabolizing Enzymes

The effect of ethanol administration on hepatic cytochrome P450 and b5 content and some monooxygenase activities is shown in Table 1. With respect to microsomes from control animals, the ethanol treatment resulted in a 2.4- and a 3-fold increase of the oxidation rates of p-nitrophenol and aniline, two substrates considered to be highly selective for P4502E1. All the other monooxygenase activities, as well as the amount of both P450 and cytochrome b5, did not exhibit any significant difference between control and ethanol-treated rat liver microsomes.

To investigate more specifically the effect of ethanol administration to Osborne-Mendel rats on the constitutive P450 isozymes, the metabolism of an endogenous substrate, such as T, was studied. Results presented in Table 2 show that no significant differences were present between control and ethanol-treated hepatic microsomes either in the total level of T-hydroxylation or in the production of any specific T-metabolite, including 16β-OH and 17 OT, which are associated, respectively, with P450 2B1/2 (the major PB-inducible P450 isoenzyme) and P450 2C11 (the most relevant constitutive P450 form in the rat liver).

The immunoblot analysis of microsomal protein Figure 1 using rabbit anti-rat P4502E1 polyclonal IgG, evidenced the absence of any reaction in control microsomes for lane A, whereas a band is present in lane B where ethanol-treated rat liver microsomes were loaded.

Effect of Ethanol on Chloroform Metabolism

The levels of total covalent binding to microsomal phospholipids due to [14C]CHCl3 reactive intermediates in ethanol-pretreated Osborne-Mendel rat liver microsomes were almost identical to those measured in microsomes from untreated rats at any tested pO2 (Figure 2).

With the transmethylation of the PL-adducts of Osborne-Mendel rat liver microsomes, it is possible to selectively quantitate the production of oxidation and reduction intermediates of CHCl3 metabolism, which exhibit a typical regioselectivity in their attack to PL. Indeed, while [14C]CHCl3 radicals, reductively produced from [14C]CHCl3, preferentially bind to PL fatty acyl chains (FC), the major product of CHCl3 oxidation, phosgene, has in PL polar heads (PH) its main target (36). In Figure 3, radioactivity associated to PH, expression of CHCl3 oxidation, measured in control and in ethanol-treated Osborne-Mendel rat liver microsomes is shown. It
appears that binding to PH increased on increasing pO2, but no differences were present between control and treated microsomes; only values measured at 1% pO2 exhibited some degree of statistical significance. The binding to FC, considered as an index of CHCl3 reduction in control and treated microsomes is shown in Figure 4. The opposite dependence on pO2 with respect to PH was evidenced: the levels of radioactivity associated to FC decreased on increasing pO2. Moreover, levels measured both in control and in treated microsomes were almost identical. The slight statistical significance of the difference between values at 5% pO2 was not considered relevant. As a consequence, the relative contribution of the two different CHCl3 metabolites to the almost similar levels of PL total covalent binding measured in different oxygenation conditions (Figure 2), markedly varied with pO2 (Figures 3 and 4). Indeed, the ratios of radioactivity associated to PH versus radioactivity to FC (PH/FC ratios) were 0.28, 0.21, 1.34, and 38.5 in control microsomes. The ratios were 0.31, 0.36, 2.77 and 4.65 in ethanol treated microsomes, at increasing pO2 from 0 to 1, 5 and 20%, respectively.

Discussion

Similar to previous findings with rabbits, (37,38) hamsters (39), and different strains of mice and rats (40,41), the present results clearly indicate that the oral administration of ethanol to Osborne-Mendel rats, resulted in the induction of P4502E1. In fact, the significant increase of the hepatic P4502E1-linked monoxygenase activities was confirmed by the immunodetection in microsomes from ethanol-treated rat liver, of a band corresponding to P4502E1. That band was absent in hepatic microsomes from control Osborne-Mendel rats.

No other forms of P450 seemed to be significantly affected by ethanol pretreatment, as demonstrated by the unchanged metabolism of either exogenous and endogenous P450 substrates.

Previous works evidenced that both P4502B1/2 and P4502E1 are involved in CHCl3 metabolism, which can therefore be affected by pretreatment with PB, alcohols and ketones (6,13–15,42).

Although our pretreatment procedure resulted in the induction of P4502E1, in hepatic microsomes from Osborne-Mendel rats, no substantial quantitative changes were detectable in chloroform metabolism, which is expressed as 14CHCl3-derived reactive intermediates covalently bound to microsomal PL (Figure 2).

Moreover, considering the typical regioselectivity in the attack to PL exhibited by oxidation and reduction intermediates of CHCl3 (36), it appeared that ethanol was also ineffective in qualitatively modifying the pattern of CHCl3 metabolism (Figures 3 and 4). Indeed, the two pathways are similarly expressed in control and ethanol-treated microsomes.

The relative contributions of P450 2B1/2 and P450 2E1 appear to be dependent on the substrate concentration. Indeed, it has been suggested (42) that at low chloroform concentrations (about 0.1 mM) its metabolism is catalyzed mainly by P450 2E1 (13,15,42), while P450 2B1/2 may be significantly responsible for CHCl3 activation at a higher haloform concentration of 5 mM (14). Data on the effects of ethanol on chloroform metabolism at high substrate concentration were only based on the detection of chloroform oxidation products (14). Our data indicate that both the oxidative and the reductive metabolism of CHCl3 are not affected by P450 2E1 at 5 mM CHCl3, a concentration at which the two pathways are expressed (21).

One of the most relevant features of the CHCl3 metabolism is that the oxygenation of the incubation mixture was of major importance in determining the oxidative and/or reductive nature of CHCl3 activation (21). Figure 2, we show that at 1 and 5% pO2, representing the physiological range of O2 tensions typical of the liver (43), the two pathways are concurrently present. It appeared also that in our in vitro conditions the shift from oxidation to reduction occurred just in this range of pO2. Indeed the relative magnitude of the binding to PH and FC was reversed mostly between 5 and 1% pO2 (Figures 3 and 4).

Rats treated with ethanol showed a higher rate of O2 consumption in the liver than control rats (44). This phenomenon may increase the state of physiological hypoxia of the liver and suggests that the consequent alteration of the delicate balance between the oxidative and the reductive activation of CHCl3 may concur in the potentiation of CHCl3 toxicity.

Figure 4. Effect of ethanol-pretreatment on in vitro covalent binding of CHCl3 metabolites to liver microsomal PL fatty acyl chains (for details see Figure 3)
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