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Liver nuclear and microsomal CYP2E1-mediated metabolism of xenobiotics in rats chronically drinking an alcohol-containing liquid diet

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In previous studies from our laboratory, the presence in highly purified liver nuclei of metabolic pathways for processing ethanol (EtOH), *N*-nitrosodimethylamine (NDMA), carbon tetrachloride and chloroform was reported. All these chemicals are known to be metabolized in liver microsomes, via cytochrome P450 2E1 (CYP2E1)-mediated processes. In the present work we checked whether rat liver nuclei from rats chronically drinking an alcohol-containing liquid diet exhibited an enhanced ability to metabolize chemicals known to require CYP2E1 participation for given metabolic transformations. The nicotinamide adenosine dinucleotide phosphate (NADPH)-requiring metabolism of *p*-nitrophenol to *p*-nitrocathecol; the activation of carbon tetrachloride to trichloromethyl radicals, covalently binding to proteins; and the ring hydroxylation of aniline and *o*-toluidine were studied. Comparison of the obtained nuclear activities against the one present in the microsomal counterpart, and their respective response of them to the EtOH inductive effect after repetitive exposure to it, was studied. The obtained results showed that rat liver nuclei exhibited less *p*-nitrophenol hydroxylase activity than microsomes, but it was inducible by repetitive alcohol drinking to equivalent levels of those of microsomes from control animals. Nuclei exhibited the ability to activate CCl_4, which was significantly enhanced by alcohol drinking. Aniline was ring hydroxylated in liver microsomes but not in nuclei from either control or EtOH-treated animals. In contrast, nuclei and microsomes metabolized *o*-toluidine to ring hydroxylated products. They are considered less toxic in nature but other authors reported a genotoxic effect for one of them. The production of the ring hydroxylated metabolites was enhanced by repetitive EtOH drinking. Results suggest that nuclear metabolism of xenobiotics might be relevant for either activations or detoxications mediated by CYP2E1 and that repetitive exposure to EtOH might significantly modulate those processes. *Toxicology and Industrial Health* 2006; **22:** 1–8.

Key words: *alcohol; aniline; carbon tetrachloride; CYP2E1; liver; N-nitrosodimethylamine; nuclear; nuclei; o-toluidine*

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Introduction

In previous studies from our laboratory, the presence in highly purified liver nuclei of an ethanol (EtOH)-metabolizing system (NEMS) was reported. This NEMS led to the production of acetaldehyde (AC), 1-hydroxyethyl (1HEt) and hydroxyl free radicals (Castro *et al.*, 1998).

These reactive EtOH metabolites proved in further studies to covalently bind to nuclear lipids and proteins (Díaz Gómez *et al*., 1999). Most of the NEMS activity was observed in the presence of nicotinamide adenosine dinucleotide phosphate (NADPH) and oxygen and was inhibited by CO, SKF525A and diethyldithiocarbamate (DDTC) (Castro *et al*., 1998). Further, repetitive administration of EtOH during 28 days of a standard Lieber & De Carli diet enhanced the NEMS activity and the ability of liver nuclei to metabolize the potent carcinogen *N*-nitrosodimethylamine (NDMA) (Díaz Gómez *et al*., 2002). These previous observations strongly suggest not only that the NEMS would be cytochrome P450 2E1 (CYP2E1)-mediated but also that liver nuclei from alcohol-exposed animals might have increased ability to either metabolically activate or detoxify xenobiotics whose biotransformation is mediated by CYP2E1.

In this respect it is important to emphasize that many environmentally relevant procarcinogens or xenobiotics are metabolized by processes involving CYP2E1 (Bolt *et al*., 2003; González, 2005; Lieber, 2004).

In the present work we attempt to provide further evidence of the presence of CYP2E1-mediated biotransformations in liver nuclei and the enhanced ability of the latter to process xenobiotics for activation or detoxication after chronic EtOH drinking. We selected for these purposes a widely used marker of CYP2E1-mediated metabolism, *p*-nitrophenol hydroxylation to *p*-nitrocatechol (Mishin *et al*., 1996). To illustrate the consequences of CYP2E1 induction in liver nuclei resulting from repetitive EtOH drinking, we selected for the present studies: 1) The metabolism of CCl_4 to the highly reactive trichloromethyl radicals that bind covalently to nuclear proteins (Castro *et al*., 1989, 1990; Díaz Gómez & Castro, 1980a; Ekström *et al*., 1989; Fanelli & Castro, 1993). 2) The metabolism of aniline to aminophenols (Gut *et al*., 1996; Koops & Coon, 1986) and that of *o*-toluidine to give ring hydroxylated derivatives (Gupta *et al*., 1987; IARC, 1982).

Comparison against the respective microsomal activities was made. This work is a report of the obtained results.

Materials and methods

Chemicals

[^{14}C]CCl_4 (specific activity 27 mCi/mmol, purity > 99% by GLC analysis) was purchased from the Radiochemical Centre (Amersham, England). Aniline, *o*-toluidine, 2-aminophenol (OAF), 4-aminophenol (PAF), 3-amino-*o*-cresol (3AOC), 2-amino-*m*-cresol (2AMC), 4-amino-*m*-cresol (4AMC), 2-amino-*p*-cresol (2APC), heptafluorobutyric anhydride (HFBA), *p*-nitrophenol, *p*-nitrocatechol, D-glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma-Aldrich (Steinheim, Germany). Aniline was distilled before use. All other chemicals were obtained from a commercial source and were of reagent grade.

Animals and treatments

Non-inbred male Sprague Dawley rats were used. The procedures used for breeding, housing and handling animals were those established by the Food, Drug and Medical Technology National Administration (ANMAT, Buenos Aires, Argentina).Sprague Dawley male rats (125–150 g body weight) were fed with a nutritionally adequate liquid diet (Lieber & De Carli standard rat diet, purchased from Dyets, Inc., Pennsylvania, USA) (Lieber & De Carli, 1982, 1989). The rats were housed in individual cages and separated into two dietary groups: the ethanol group (EtOH) and control group. Both groups were pair fed with the same diet except that in the control group EtOH was isocalorically replaced with carbohydrate (dextrin maltose). The liquid diet used provided 1 kcal/mL where 35% of the total calories derived from fat, 47% from carbohydrate and 18% from protein. In the EtOH-treated animals, EtOH provided 36% of the calories replacing isocalorically carbohydrate. Feeding of the control and EtOH

diet was continued for 28 days. The amount of EtOH was started with 30 g/L of the liquid diet for the first two days, 40 g/L for the subsequent two days, followed by the final formula containing 50 g/L.

Isolation of highly purified nuclear preparations

Highly purified nuclei were obtained as previously described (Díaz Gómez *et al*., 1999; Viviani *et al*., 1978). Briefly, the liver homogenate in 0.25 M sucrose in TKM buffer (50 mM Tris–HCl, 5 mM $MgCl_2$ and 2.5 mM KCl), pH 7.5, was passed through a 100-mesh nylon cloth and centrifuged at $1000 \times g$ for 20 min. After washing the pellet twice by resuspending with 0.25 M sucrose-TKM and centrifugation at $1000 \times g$ for 5 min, the crude preparation was resuspended in 2.2 M sucrose-TKM, layered on 2.3 M sucrose-TKM and centrifuged for 20 min at $80\,000 \times g$. The pellet was gently rinsed with 0.25 M sucrose-TKM and resuspended in 2.2 M sucrose-TKM and the ultracentrifugation step was repeated. Finally, the pellet was washed with 0.25 M sucrose-TKM. The purity of nuclei was assessed on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase) and by phase contrast microscopy in order to obtain an assessment of the purity of nuclear preparations (Figure 1).

Isolation of the microsomal fraction

Livers were homogenized via Teflon-glass Potter-Elvehjem homogenizer with 4 volumes of 1.15% (w/v) KCl. The homogenates were centrifuged at $9000 \times g$ for 20 min. The resulting supernatants were centrifuged at $100\,000 \times g$ for 1 h and the microsomal pellets were recovered (Castro *et al*., 2002).

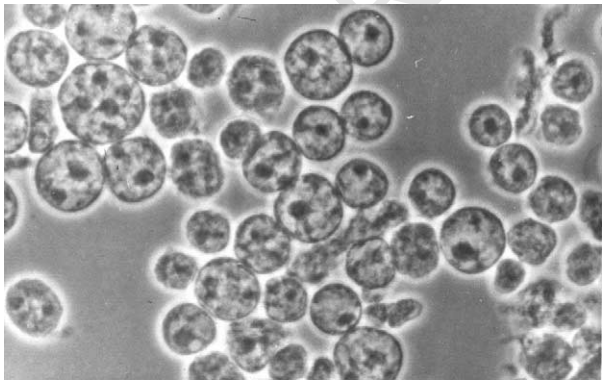

Figure 1. Contrast phase micrograph from a highly purified nuclei preparation. ×1800.

p-Nitrophenolhydroxylase activity in rat liver nuclei and microsomes

p-Nitrophenol hydroxylase activity was determined essentially as described by Mishin *et al*. (1996), with minor modifications. Microsomes (~1 mg protein/mL) or nuclei (~3.85 mg protein/mL) were incubated for 30 min and 1 h respectively, at 37°C in 50 mM phosphate buffer, pH 7.4, containing 100 μM *p*-nitrophenol and NADPH-generating system (26 mM $NADP^+$, 66 mM D-glucose-6-phosphate, 66 mM $MgCl_2$, 40 U/mL glucose-6-phosphate dehydrogenase), with a final volume of 0.5 ml. Blanks were also run by omitting the NADPH-generating system from the incubation mixture. The reaction was terminated by addition of 100 μL of trichloroacetic acid (TCA) and centrifuged for 10 min at $10\,000 \times g$. The supernatant was used for the HPLC determination.

HPLC procedure

Analysis of the metabolite 4-nitrocatechol was performed by HPLC (Hewlett Packard 1090) on a reversed phase C18 column (HP ODS Hypersil 5 μm, 200 × 2.1 mm). An ESA Coulochem II electrochemical detector was used, equipped with an 5011A analytical cell, set at 700 mV and ultraviolet absorbance was also monitored at 345 nm. Elution was isocratical with a mobile phase consisting of 25% acetonitrile in 0.1% TCA. AQ3

Covalent binding of [^{14}C] from [^{14}C]CCl_4 to proteins in rat liver nuclei and microsomes

Incubation mixtures contained: rat liver nuclei (4.0–4.5 mg protein/mL) in 0.25 M sucrose in TKM buffer, pH 7.4, or microsomes (2.5–3.0 mg protein/mL) in 0.17 M KH_2PO_4 buffer, pH 7.2, [^{14}C]CCl_4 (0.2 mM, 0.17×10^6 dpm/mL) and NADPH-generating system when indicated. Incubation was conducted for 1 h for the case of nuclei and 15 min for microsomes at 37°C and the reaction was stopped with 10% TCA. The covalent binding (CB) to proteins was determined in the acid

insoluble precipitate as described (Díaz Gómez & Castro, 1980a). Proteins were precipitated and washed with TCA, delipidated and dried by successive washings with the use of a mixture of EtOH:ether:chloroform (2:2:1), acetone and ether. Finally they were dissolved in concentrated formic acid and counted with a butyl cellosolve–PPO mixture.

Aniline and *o*-toluidine aromatic hydroxylation in rat liver nuclei and microsomes

Incubation mixtures containing highly purified nuclei (5.1 ± 0.4 mg prot/mL) or microsomes (5.5 ± 0.1 mg prot/mL), NADPH-generating system (0.45 mM $NADP^+$, 4 mM DL-isocitric acid trisodium salt and 0.25 U isocitric dehydrogenase) and 100 μM aniline or *o*-toluidine in STKM buffer (0.25 M sucrose, 50 mM Tris–HCl, 2.5 mM KCl, 5 mM $MgCl_2$) pH 7.5 for nuclei or 100 mM phosphate buffer pH 6.9 for microsomes, in a final volume of 3 mL, were conducted for 1 h at 37°C under an air atmosphere. After incubation, an internal standard solution (3AOC for aniline or 2APC for *o*-toluidine) was added and the volume was extracted with 800 μL of toluene and centrifuged.

The organic layer (500 μL) was derivatized with 10 μL HFBA at room temperature for 15 min (Jodynis-Liebert & Bennasir, 2000; Stillwell *et al*., 1987). To remove the excess of HFBA, 2 mL of 100 mM phosphate buffer was added. Then samples were shaken for 10 min, centrifuged and analysed by capillary gas chromatography using an electron capture detector (ECD). Analysis of hydroxylated amines was performed with a Hewlett-Packard 5890 series II gas chromatograph equipped with a ^{63}Ni ECD. The detector was operated at 280°C with UHP nitrogen (60 mL/min) as the make-up gas. Injector temperature was 250°C, operated in the splitless mode. Column: HP-5 (cross-linked 5% PhMe silicone), 25 m × 0.32 mm, film thickness 0.52 μm. UHP helium was used as the carrier at 3 mL/min. Temperature programme was as follows: 3 min at 140°C, then heating to 250°C at 10°C/min. Retention time of the compounds analysed (identified by GC-MS as their bis-(heptafluorobutyril) derivatives) were: OAF, 4.25 min; PAF, 5.27 min; 3OAC, 5.63; 2AMC, 3.82 min; 2APC, 5.29 min; and 4AMC, 5.98 min. Quantitative determinations were made only for OAF and 2AMC because of the low peak levels detected in the case of PAF and 4AMC. The response was linear in the range studied (2–40 pg/μL). Linear regression equations were as follows: OAF, $y = 0.0057x - 0.0065$. The correlation coefficient was $r^2 = 0.99$ (2–28 pg/μL); 2AMC, $y = 0.0042x - 0.0021$; $r^2 = 0.97$ (2–40 pg/μL). Detection limit was 1 pg/μL.

Protein concentrations

Protein concentrations were determined by the method of Lowry *et al*. (1951), using bovine serum albumin as standard.

Statistics

The significance of the difference between two mean values was assessed by unpaired *t*-test (Student's *t*-test) (Gad, 2001). Calculations were performed using GraphPad Software. Differences were considered significant when $P < 0.05$.

Results

p-Nitrophenol hydroxylase activity in liver nuclei and microsomes from rats treated with EtOH liquid diet

Results obtained for *p*-nitrophenol hydroxylation as determined by HPLC with electrochemical detection can be seen in Table 1. Nuclei from rats treated with EtOH liquid diet showed a significant increase in the formation of *p*-nitrocatechol as compared to controls. The same is observed in

Table 1. *p*-Nitrophenol hydroxylase activity in liver nuclei and microsomes from rats treated with EtOH liquid diet

Subcellular fraction[a]	Formation of 4-nitrocatechol (pmol/min per mg protein × 10^{-2})[b]	
	Control	EtOH treated
Nuclei	1.10 ± 0.06	3.80 ± 0.58[c]
Microsomes	4.95 ± 0.50	25.06 ± 3.26[c]

[a]Microsomes (~1 mg prot/mL) or nuclei (~3.85 mg prot/mL) were incubated in 50 mM phosphate, pH 7.4, 2 mM NADPH and 100 μM *p*-nitrophenol at 37°C for 30 min and 1 h respectively. Then they were processed to determine enzymatic activity by HPLC with electrochemical detection as described in Methods.
[b]Results are the means of three separate determinations from three different livers.
[c]$P < 0.05$ (EtOH versus control).

microsomes, although in an even much greater magnitude.

Covalent binding of [^{14}C] from [^{14}C]CCl_4 to proteins from liver nuclei and microsomes from rats treated with EtOH liquid diet

CB was measured in nuclear and microsomal proteins from control and EtOH-treated rats fed with the Lieber & De Carli diet. Both nuclei and microsomes were able to bioactivate CCl_4 to metabolites that bound covalently to proteins and this metabolic transformation was significantly increased in the presence of NADPH in control rats (Table 2). When CB was measured in proteins from nuclei and microsomes from EtOH-treated rats, the ability for CCl_4 activation to reactive species able to react covalently with proteins was enhanced in a highly significant manner in the presence of NADPH compared to the untreated rats (Table 2).

Aniline and *o*-toluidine aromatic hydroxylation in liver nuclei and microsomes from rats treated with EtOH liquid diet

Results showed that rat liver microsomes from control rats were able to biotransform aniline to OAF and PAF (Figure 2a), and *o*-toluidine to 2AMC and 4AMC, in the presence of NADPH (Figure 2b). Nuclei only showed ability to oxidize *o*-toluidine (Table 3). Only OAF (for aniline) and 2AMC (for *o*-toluidine) were quantified in microsomes and nuclei. PAF and 4AMC were not quantified because they gave small peak areas. Metabolite formation in rats repeatedly treated with EtOH (in the presence of NADPH) was significantly induced with respect to controls (Table 3).

In EtOH-treated rats, biotransformation of *o*-toluidine to its metabolite 2AMC in liver rat microsomes was increased up to six times with respect to control rats. In the case of aniline, the formation of OAF was increased by a factor of two.

Table 2. Covalent binding of [^{14}C] from [^{14}C]CCl_4 to proteins from liver nuclei and microsomes from rats treated with EtOH liquid diet

Experimental condition[a]	Covalent binding (nmol $CCl_4 \times 10^{-1}$/mg protein)	
	Control	EtOH treated
Nuclei		
−NADPH	4.99 ± 0.08	5.24 ± 0.09
+NADPH	8.97 ± 0.50[b]	16.47 ± 0.80[b,c]
Microsomes		
−NADPH	5.50 ± 0.06	6.50 ± 0.13
+NADPH	31.28 ± 1.98[b]	67.76 ± 3.12[b,c]

[a]Incubation mixtures contained: rat liver nuclei (4.0–4.5 mg protein/mL) in 0.25 M sucrose in TKM buffer, pH 7.4, or microsomes (2.5–3.0 mg protein/mL) in 0.17 M KH_2PO_4 buffer, pH 7.2, [^{14}C]CCl_4 (0.2 mM, 0.17×10^6 dpm/mL) and NADPH-generating system when indicated. Incubation was conducted for 1 h for the case of nuclei and 15 min for microsomes, at 37°C and the reaction was stopped with 10% TCA. Covalent binding was determined as indicated in Methods. Results are the means of three determinations.
[b]$P < 0.05$ (+NADPH versus −NADPH).
[c]$P < 0.05$ (EtOH versus control).

Discussion

In agreement with previous studies from other laboratories, we observed that repetitive alcohol drinking is able to induce liver microsomal *p*-nitrophenol hydroxylase activity (Mishin *et al*., 1996).

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol has been widely used as a marker for CYP2E1 activity (Mishin *et al*., 1996). Now we report that highly purified liver nuclei also exhibit *p*-nitrophenol hydroxylase activity. This nuclear activity is about seven times smaller than in the microsomal fraction and also inducible by repetitive alcohol drinking. The inductive effect of alcohol drinking enhances the liver nuclear CYP2E1 activity to levels closely similar to those present in control microsomes. These results and those previously reported by our laboratory showing that chronic alcohol drinking also induces the liver nuclear metabolism of EtOH to AC and the one of NDMA to formaldehyde and to methylcarbonium ions that bind to nuclear proteins (Díaz Gómez *et al*., 2002), further indicate that liver nuclei are able to perform CYP2E1-mediated transformations. In effect, both alcohol and NDMA are well known substrates for CYP2E1 metabolic transformations (Bolt *et al*., 2003; González, 2005; Guengerich, 1995; Lieber, 2004).

An interesting point of these two latter cases is that nuclear activation opened the possibility for very reactive metabolites (eg, a free radical or a carbonium ion) produced so close in molecular terms to critical targets such as DNA; nuclear proteins or lipids to reach them and lead to harmful

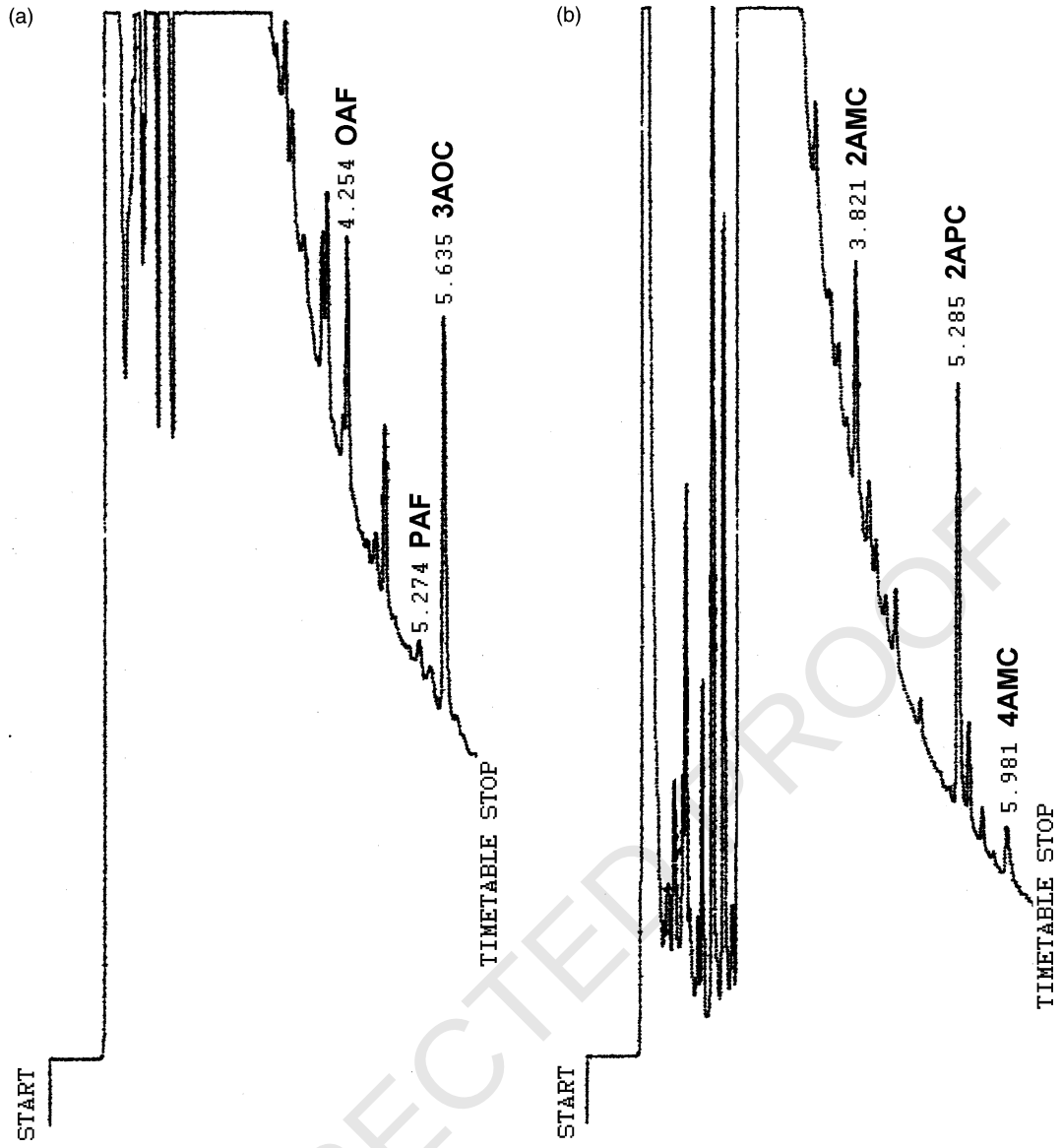

Figure 2. (a) Gas chromatogram (ECD detection) obtained from a sample of incubation containing microsomes, NADPH-generating system and 100 μM aniline, after derivatization with HFBA. OAF, 2-aminophenol; PAF, 4-aminophenol; 3AOC, 3-amino-*o*-cresol (internal standard). (b) The same as in (a) but in the presence of 100 μM *o*-toluidine. 2AMC, 2-amino-*m*-cresol; 4AMC, 4-amino-*m*-cresol; 2APC, 2-amino-*p*-cresol (internal standard).

consequences. CB to nuclear proteins and lipids was shown to occur during liver nuclear activation of alcohol to AC and 1HEt radicals (Díaz Gómez *et al*., 1999). Similarly, CB of reactive moieties to nuclear proteins was observed during nuclear metabolism of NDMA to formaldehyde and methylcarbonium ions (Díaz Gómez *et al.*, 2002).

A wide spectrum of environmentally important chemicals are known to be metabolized by liver microsomal CYP2E1 to either more toxic reactive metabolites or, conversely, to less harmful metabolites and excellent reviews are available on the subject (Bolt *et al*., 2003; González, 2005; Guengerich, 1995; Lieber, 2004). An equivalent amount of information is not available for the case of the liver nuclear counterpart. In the past our laboratory reported the liver nuclear biotransformation of carbon tetrachloride and chloroform to reactive metabolites that bind covalently to either DNA and/or nuclear proteins and lipids (Castro *et al*., 1989, 1990; Díaz Gómez & Castro, 1980a,b,c; Fanelli & Castro, 1993). Later studies by others gave evidence that the liver microsomal metabolism of both haloalkanes is mediated by CYP2E1

Table 3. Aniline and *o*-toluidine aromatic hydroxylation in liver nuclei and microsomes from rats treated with EtOH liquid diet

Experimental condition[a]	Control	Alcohol treated
Aniline (pmol OAF/mg prot)		
Microsomes	44.37 ± 1.79	96.09 ± 21.06[b]
Nuclei	n.d.	n.d.
o-Toluidine (pmol 2AMC/mg prot)		
Microsomes	17.61 ± 4.01	135.65 ± 24.02[b]
Nuclei	n.d.	23.30 ± 4.04

[a]Incubation mixtures containing microsomal or nuclei preparations, NADPH-generating system and 100 μM aniline or *o*-toluidine, were conducted for 1 h at 37°C. Each result is the mean of three separate samples. Samples were analysed in the GC-ECD by triplicate. n.d., not detected.
[b]$P < 0.05$ (EtOH versus control).

(Morimoto *et al*., 1995). Further, in the case of CCl_4, alcohol pretreatment remarkably stimulates its toxicity (Hasumura *et al*., 1974), with perivenular predominance, which can be explained by selective presence and induction of CYP2E1 in that zone of the liver lobule (Ingelman-Sundberg *et al*., 1988; Tsutsumi *et al*., 1989).

In the present study we report that the activation of CCl_4 is enhanced in rats repetitively drinking alcohol not only in liver microsomes but also in the liver nuclear counterpart. The relevance of this is that the trichloromethyl radical metabolite that binds covalently to nuclear proteins is also enhanced because of the chronic alcohol drinking. A highly reactive moiety such as $^{\bullet}CCl_3$ would not be able to migrate, even in molecular terms, from the endoplasmic reticulum to nuclear targets. Consequently, the inductive action of alcohol drinking on the nuclear CYP2E1-mediated activation pathway leading to CB to nuclear proteins may be of relevance.

In fact, previous studies from our laboratory showed a better correlation between that CB to nuclear proteins than the one to DNA in relation to the different species susceptibility to CCl_4-induced liver cancer (Castro *et al.*, 1989).

Liver microsomes are known to be able to metabolize *o*-toluidine to ring hydroxylated derivatives (Danford, 1991). However, liver nuclei also showed considerable ability for metabolizing xenobiotics via CYP2E1-mediated biotransformations to less toxic metabolites. This was suggested by our studies on *o*-toluidine ring hydroxylation. In our present studies both liver microsomes and nuclei exhibited ring hydroxylating capacity and response to the inductive effect of chronic alcohol drinking. As expected, microsomal capacity was higher than that of nuclei. We failed to detect an equivalent nuclear activity for aniline ring hydroxylation despite the very sensitive GC/ECD method employed. However, the relevance or not of these nuclear pathways of potential detoxication remains to be challenged. In effect, some data from others indicate that the hydroxylated metabolite 4AMC seems to have a potential for genotoxicity (Ohkuma *et al.*, 1999). Consequently, we consider of interest our observations about the existence of an alcohol-inducible metabolic pathway so close to significant cellular targets such as DNA, nuclear proteins or lipids. The *N*-hydroxylation pathway, which is known to be catalyzed by P450, is not under consideration in the present work. The possibility for EtOH to induce these pathways and their role in amine carcinogenicity is at present under study in our laboratory.

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