Teratogenesis, Carcinogenesis, and Mutagenesis 22:335–341 (2002)

Rat Ventral Prostate Microsomal Biotransformation of Ethanol to Acetaldehyde and 1-Hydroxyethyl Radicals: Its Potential Contribution to Prostate Tumor Promotion

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Rat ventral prostate microsomal fraction was able to biotransform ethanol to acetaldehyde and 1-hydroxyethyl radicals (1HEt) in the presence of NADPH and oxygen. The enzymatic processes involved were not inhibited by desferrioxamine, CO, SKF 525A, 4-methylpyrazole, or polyclonal antibody against P450 reductase but they were significantly inhibited by diethyldithiocarbamate, 2-mercapto-1-methylimidazol, thiobenzamide, or diphenyleneiodonium chloride. Results would suggest the partial participation in these ethanol bioactivation processes of flavin containing monooxygenase (FMO) and/or other flavin dependent oxidases/peroxidases and of a non-iron metal-containing enzymes. Acetaldehyde and free radicals production by prostate microsomal fraction might potentially contribute to tumor promotion in heavy alcohol drinkers. *Teratogenesis Carcinog. Mutagen. 22:335–341, 2002.* © 2002 Wiley-Liss, Inc.

Key words: alcohol; spin trapping; free radicals; GC-MS; cancer

INTRODUCTION

Epidemiological studies on a potential correlation between alcohol drinking and prostate cancer were recently reviewed and their authors found no convincing

Contract grant sponsor: Agencia de Promoción Científica y Tecnológica, Argentina.

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/tcm.10028

correlation between alcohol consumption and prostate cancer incidence [1,2]. However, the authors did mention six out of thirty-two studies analyzed where positive correlations were reported. Most negative studies, however, did not assess the risk of heavy drinking, where there has been some suggestion of increased risk [3–6].

There is a need for research on possible mechanisms by which ethanol might theoretically promote the development of cancer in prostatic tissues. According to present views, ethanol ability to promote cancer was linked to its biotransformation to acetaldehyde and free radicals, to its ability to modulate procarcinogens biotransformation, or to its impairing action on immune function or depress levels of DNA repair enzymes [7,8].

In a recent study from our laboratory, we reported that rat ventral prostate cytosolic xanthine oxidase/xanthine dehydrogenase enzymes were able to activate ethanol to acetaldehyde and to 1-hydroxyethyl radicals (1HEt) [9]. In the present work, the ability of rat ventral prostate microsomal fraction to biotransform ethanol to reactive metabolites is discussed and initial efforts to characterize the enzymes involved are reported.

MATERIAL AND METHODS

Chemicals

Absolute ethanol (analytical grade) was from Sintorgan (Argentina). N-tert-Butyl-(-phenylnitrone (PBN) and the drugs tested for their effects on the metabolism of ethanol were from Sigma Chemical Co. (St. Louis, MO): SKF 525A, 4-methylpyrazole (4MP), thiobenzamide (TBA), N,N-diethyldithiocarbamic acid sodium salt (DDTC), 2-mercapto-1-methylimidazole (MMI), NAD^+, $NADP^+$, acetylsalicylic acid (ASA), desferrioxamine mesylate (DFA), 3-amino-1,2,4-triazole (AT), diphenyleneiodonium chloride (DPI), and indomethacin (IM). Nitrogen (ultra high purity) was from AGA (Argentina) and carbon monoxide was from Matheson Co (Newark, CA). Both gases were further deoxygenated by bubbling through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1 N NaOH. The polyclonal antibody against rat liver microsomal NADPH P450 reductase was from Gentest Corp (Woburn, MA).

Animals and Treatments

Non-inbred male Sprague Dawley rats (220–260 g, age range: 8–9 weeks) were used. The animals were starved for 12–14 h before sacrifice. Water was available ad libitum. Animals were killed by decapitation and their ventral prostates were rapidly excised and processed. Microsomes were obtained as previously described and were essentially free from cross contamination [10]. For the experiments involved in detection of 1HEt free radicals, microsomal pellets were resuspended in buffer containing 0.5 mM DFA and recentrifuged in order to remove traces of free iron.

Ethanol Biotransformation to Acetaldehyde in the Microsomal Fraction

Preparations containing microsomes (1.84 ± 0.50 mg of microsomal protein/ml), NADPH generating system (0.45 mM $NADP^+$, 4 mM d,l-isocitric acid trisodium salt, and 0.25 units of isocitric dehydrogenase), and 0.21 M ethanol in STKM buffer (0.25 M sucrose/50 mM Tris-HCl, pH 7.5/2.5 mM KCl/5 mM $MgCl_2$), 3 ml final volume, were incubated for 1 h at 37°C under different atmospheres [air, nitrogen, $CO:O_2$ (80:20 v/v)]. Incubations were performed in aluminium-sealed-neoprene-septum-stoppered glass vials (15 ml). In the case of the antibody against P450 reductase, incubation conditions were essentially as described by Diaz Gomez et al. [11] and a control reaction was run using normal serum. The reaction was stopped by placing the vials on ice. After adding 1 ml of saturated NaCl solution, samples were maintained at 40°C for 10 min and an aliquot (100 μl) of the head space analyzed by GC-FID. Chromatographic conditions were the following: column, Poraplot Q, 25 m × 0.53 mm i.d. (Chrompack, Netherlands); temperature 140°C isothermal, injection port temperature, 150°C, FID: 200°C [12].

Biotransformation of Ethanol to 1-Hydroxyethyl Radical by Rat Prostate Microsomes

The spin adduct of the 1HEt radical was detected by the method described previously [13,14]. Purified microsomes (1.3–2.3 mg protein per ml) were added to NADPH generating system, 0.15 M $MgCl_2$, 24 mM PBN, and 0.21 M ethanol in 0.25 M STKM. After 1 h at 37°C, the volume (3 ml) was extracted with 500 μl toluene, centrifuged, and the organic layer evaporated under nitrogen. The residue was silylated with BSTFA and analyzed by GC/MS. Chromatographic conditions were at follows: column, 5% phenylmethyl silicone, 12 m × 0.2 mm i.d., programmed from 100 to 300°C at a ramp of 10°C/min. Injection port was at 250°C and transfer line to MS, 300°C. Selected ion monitoring (SIM) of mass spectra was employed to increase sensitivity. Selected masses were 250 (M-·$CHCH_3OTMS$) and 194 (m/z 250-C_4H_8).

Statistics

The significance of the difference between two mean values was assessed by the Student's t-test [15].

RESULTS

Ethanol biotransformation to acetaldehyde in the ventral prostate microsomal fraction. Results on acetaldehyde levels for incubations containing microsomes are summarized in Table I. The reaction was sensitive to heating 5 min at 100°C. Replacing air by a 80:20 mixture of CO to O_2 atmosphere or including in the mixture 1 mM SKF 525A were not able to appreciably decrease aerobic biotransformation. Indeed, SKF 525A caused a significant increase in response. Other chemicals 4MP and DDTC, known for their inhibitory effect on P450 (CYP2E1) mediated reactions, were tested [16]. Only the latter compound appeared to inhibit acetaldehyde production. Its effect would not be related necessarily to inhibition of CYP2E1 in light of the lack of response of the others. The antibody against liver microsomal P450 reductase was not able to inhibit the biotransformation of ETOH to acetaldehyde. Acetaldehyde production was strongly dependent on the presence of oxygen. Inhibitors of prostaglandin endoperoxide synthase such as ASA or IM [17]

TABLE I. Ethanol Biotransformation to Acetaldehyde by Ventral Prostate Microsomes

Experimental[a,b]	Acetaldehyde (ng)/protein (mg) +NADPH	−NADPH
Air	38.2±4.4	14.4±2.7
Heated (100°C, 5 min)	10.4±1.8	6.4±0.5
$CO:O_2$ (80:20)	38.0±2.7	15.7±1.9
1 mM SKF 525A	51.9±1.9[c]	18.3±1.0
5 mM 4MP	39.3±0.6	15.5±0.3
1 mM DDTC	8.5±1.2	5.6±0.3
Nitrogen	2.6±0.4	3.8±0.1
1 mM ASA	42.4±1.0[d]	14.2±2.4
30 μM IM	38.4±0.9[d]	13.5±0.3
10 mM AT	40.4±0.9[d]	13.5±1.0
1 mM MMI	23.2±0.5[c]	13.0±1.1
1 mM TBA	22.0±0.2[c]	11.2±1.0
10 μM DPI/air	12.7±0.1	8.0±0.7
10 μM DPI/nitrogen	3.2±0.2	3.7±0.9
1 mM DFA	36.5±1.7	9.7±0.2
Polyclonal antibody against P450 red (air)	39.4±0.9	36.1±1.3[e]
Polyclonal antibody against P450 red (nitrogen)	8.1±0.3	3.6±0.1[e]

[a]Incubation mixtures containing microsomal preparations (1.84±0.50 mg of microsomal protein/ml), NADPH generating system, and 0.21 M ethanol were conducted for 1 h at 37°C. Incubations containing the polyclonal antibody against P450 reductase were performed as previously described [11]. Acetaldehyde was measured in the head space of each sample after adding 1 ml NaCl saturated solution. (See Methods for details). Each result is the mean of three separate lots of pooled prostate samples.
[b]ASA, acetylsalicylic acid; AT, 3-amino-1,2,4-triazole; DDTC, N,N-diethyldithiocarbamic acid sodium salt; DFA, desferrioxamine mesylate; DPI, diphenyleneiodonium chloride; IM, indomethacin; MMI, 2-mercapto-1-methylimidazole; 4MP, 4-methylpyrazole; TBA, thiobenzamide.
[c]$P<0.05$ when compared to "Air+NADPH."
[d]$P>0.05$ when compared to "Air+NADPH."
[e]Corresponding to the antiserum, in the presence of NADPH.

or of catalase, like AT [16,17] did not cause any significant depletion in the production of acetaldehyde.

The role of non heme iron in the metabolism to acetaldehyde was checked by the use of desferrioxamine (DFA) but, under air, no effect was observed.

MMI, TBA, and 10 μM DPI were tested as potential inhibitors of biotransformation. In both cases significant differences were observed, when compared to the acetaldehyde formed under air+NADPH.

1-Hydroxyethyl Radical Determination in the Ventral Prostate Microsomal Fraction

Figure 1a shows the capillary GC analysis with TIC detection of reaction products when free radicals were derived from ethanol biotransformation by ventral prostate microsomes in the presence of the spin trap PBN. The spin adduct of the 1HEt radical was detected (Fig. 1a) when NADPH was present and only traces were observed when NADPH was absent (Fig. 1b). In addition, two peaks (A and B in Fig. 1a) due to the interaction between hydroxyl radicals and PBN were observed. No ethanol was necessary for them to be formed (Fig. 1c). These compounds were

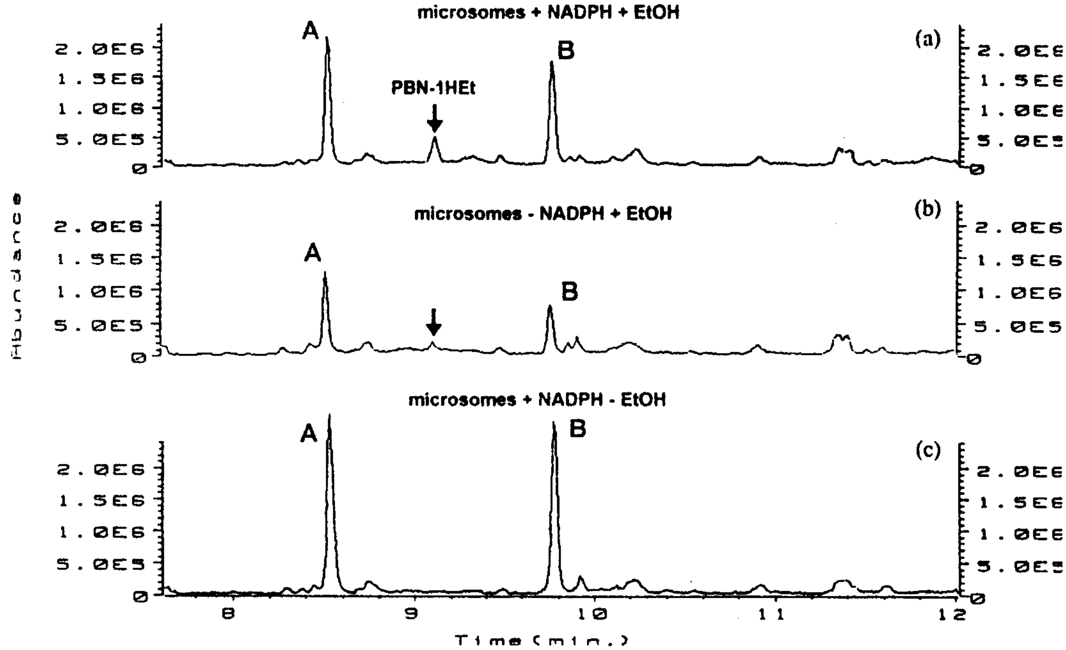

Fig. 1. **a:** Selected-ion current profile obtained from GC-MS-SIM analysis of a sample of incubation that contains microsomes, NADPH, ethanol, and PBN, after trimethylsilylation. Masses selected for SIM were 250 and 194 (see Methods for details). Peaks: PBN-1HEt, 1-hydroxyethyl adduct of PBN; A and B, two isomers of aromatic-hydroxylation derivatives of PBN. **b:** The same as in **a** but in the absence of NADPH. **c:** The same as in **a** but in the absence of ethanol.

previously observed by us to be formed in other biological situations and identified as aromatic-hydroxylation derivatives of PBN [14].

DISCUSSION

The present results provide evidence that rat prostate microsomes have enzymatic NADPH-dependent pathways of ethanol biotransformation to acetaldehyde. Those processes do not appear to be mediated by cytochrome P450 (P450) enzymes (e.g., CYP2E1 or others) as has been shown to occur in liver [16]. General inhibitors of P450 biotransformations such as CO or SKF 525A [18] did not inhibit NADPH-mediated oxidation of ethanol. In contrast to previous findings in the liver [16] specific inhibitors of CYP2E1, such as 4MP, did not decrease ethanol biotransformation in rat prostate microsomes.

These processes require oxygen from air since they do not proceed under nitrogen. The lack of inhibition by DFA excludes the possibility that an iron-catalyzed reaction was involved as an artefact [19]. Potent inhibitors of prostaglandin endoperoxide synthase, such as ASA or IM [17], or of catalase, like AT [16], were not inhibitory in this case and that excludes their participation in biotransforming ethanol to acetaldehyde in prostate microsomes. DPI, a known inhibitor of flavoprotein catalyzed reactions [20,21], appeared to completely inhibit the prostate microsomal oxidation of ethanol implicating the involvement of flavin monooxygenases such as FMO. This conclusion was supported by inhibitory effects of MMI and TBA on acetaldehyde formation, since both of these are relatively specific competitive inhibitors of FMO [22,23]. FMO participation in this biotransformation, even when significant, cannot account for all the aerobic NADPH-flavoprotein that requires ethanol metabolism. Another enzyme possibly involved in ethanol oxidation could be cytochrome P450 reductase. This enzyme is able to promote the oxidation of ethanol to acetaldehyde in a NADPH and oxygen-independent process even in the absence of P450 and also under air via generation of oxygen reactive species [11]. In the presence of the P450, the biotransformation is significantly enhanced [24]. However, ethanol oxidation was not inhibited by the polyclonal antibody against P450 reductase and consequently the nature of the flavoenzyme-mediated additional bioactivation pathway not related to FMO remains to be established.

Besides the bioactivation of ethanol to acetaldehyde by the rat ventral prostate microsomes, formation of other extremely reactive moieties was found. They are hydroxyl and 1HEt radicals. Their formation cannot be mediated by the microsomal flavoprotein FMO. The reactive moiety in this enzyme is not a free radical but a FAD-hydroperoxide [22,23] and consequently the production of these radicals might not be explained via FMO participation. The process leading to their formation remains to be established.

Concerning the toxicological relevance of the present studies, it is of interest to point out that production of acetaldehyde and free radicals might be of some relevance to prostate cancer induction observed in heavy alcohol drinkers [3–6]. Acetaldehyde is a known mutagen and carcinogen [25,26] and reactive oxygen species, free radicals, and the oxidative stress potentially that result from them were postulated to have cancer promotion effects [7,8,27–29]. The presently described microsomal ethanol bioactivation system and the recently reported xanthine oxidoreductase mediated cytosolic system [9] might be mechanistical clues that link heavy alcohol drinking and prostate cancer induction observed in some studies.

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