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Rat Ventral Prostate Xanthine Oxidase Bioactivation of Ethanol to Acetaldehyde and 1-Hydroxyethyl Free Radicals: Analysis of Its Potential Role in Heavy Alcohol Drinking Tumor-Promoting Effects

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The ability of the ventral prostate cytosolic fractions to biotransform ethanol to acetaldehyde and 1-hydroxyethyl (1HEt) radicals was tested. Acetaldehyde formation was determined by GC-FID analysis in the head space of incubation mixtures. 1HEt was determined by spin trapping with PBN followed by extraction, silylation of the adduct and GC-MS of the product. Prostate cytosol was able to biotransform ethanol to acetaldehyde in the presence of NADH, hypoxanthine, xanthine, caffeine, theobromine, theophylline, and 1,7-dimethylxanthine but not in the presence of N-methylnicotinamide. All these biotransformations were inhibited by allopurinol and were sensitive to heating for 5 min at 100°C. The biotransformation of ethanol to acetaldehyde in the presence of purines as cosubstrates was accompanied by the formation of hydroxyl and 1HEt radicals as detected by GC-MS, and the process was inhibited by allopurinol. Results suggest that prostate cytosolic xanthine oxidase is able to bioactivate ethanol to acetaldehyde and free radicals. The potential of these processes to be involved in tumor-promoting effects of heavy alcohol drinking in conjunction with high meat and/or purines consumption is analyzed. Multifactorial epidemiological studies considering that possibility might be convenient. *Teratogenesis Carcinog. Mutagen. 21:109–119, 2001.* © 2001 Wiley-Liss, Inc.

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INTRODUCTION

Prostate cancer is the ninth most common cancer in the world and the leading cancer among men in the US [1,2]. Risk factors analyzed that evidence possible increased risk include total fat, saturated animal fat, milk, and dairy products. Evidence for correlation with high energy intake was considered insufficient and no relationship was reported for the incidence of prostate cancer with high body mass, vitamin C consumption, coffee or tea drinking, and alcohol consumption [1,2]. The epidemiological studies of alcohol and prostate cancer received particular attention and were recently reviewed [3,4]. The authors found no convincing association between alcohol consumption and prostate cancer incidence [3,4]. Only 6 out of 32 studies analyzed reported a positive correlation [5–11]. The results of those studies with positive findings were considered not generalizable in those reviews because they were performed in special populations (e.g., heavy drinkers) or too incompletely described to warrant consideration [3,4]. A rationale for continuing research in this area was proposed as potentially coming from considerations of biologic plausibility and the related but weaker criteria of analogy. Basically, the authors considered that there is a critical need for research that evidences the potential of alcohol to exert actions on prostate qualitatively similar to those already known to play a role for the case of alcohol consumption promotion of cancer incidence in other locations (e.g., liver, aerodigestive tract, etc.) [3,4]. Acetaldehyde is a known carcinogenic chemical [12,13] and free radicals are known to be involved in cancer promotion by ethanol (EtOH) [14,15]. In the present study the ability of rat ventral prostate to biotransform ethanol to acetaldehyde and 1-hydroxyethyl (1HEt) free radicals in a xanthine oxidase mediated process is reported.

MATERIAL AND METHODS

Chemicals

Absolute ethanol (analytical grade) was from Sintorgan (Argentina).

N-t-butyl-α-phenylnitrone (PBN) and the drugs tested on their effects on the metabolism of ethanol (hypoxanthine, xanthine, caffeine, theobromine, theophylline, allopurinol, and dimethyl sulfoxide (DMSO), desferrioxamine mesylate, etc.) were from Sigma Co (St. Louis, MO).

Animals and Treatments

Non-inbred male Sprague Dawley rats (220–260 g) 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. Purified cytosolic fractions were obtained as previously described and are essentially free from cross contamination [16]; 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the homogenizing buffer in order to avoid the irreversible conversion (by proteolisis) of xanthine dehydrogenase (XDh) to xanthine oxidase (XO). We did not use dithioerythritol (DTE) in the cytosol preparation procedure because in that case conversion to the XDh form is nearly quantitative [17].

Ethanol Biotransformation to Acetaldehyde in the Cytosolic Fraction

Incubation mixtures containing purified cytosol (12.4 ± 3.0 mg protein per ml) and 0.28 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 conducted for 1 h at 37°C under air atmosphere. In order to test the presence of the XO enzymatic system, hypoxanthine, xanthine (0.25 mM) and 0.15 mM allopurinol were employed in incubations. Incubations were performed in aluminum-sealed-neoprene-septum stoppered glass vials (15 ml). Reaction was interrupted by placing in the cold. After adding 1 ml of saturated NaCl solution, samples were thermostatized at 40°C for 10 min and an aliquot (100 μl) of the head space analyzed by GC-FID.

Chromatographic conditions were column, Poraplot Q, 25 m × 0.53 mm i.d. (Chrompack, Netherlands); temperature 140°C isothermal, injection port temperature: 150°C, FID: 200°C [18,19].

1-Hydroxyethyl Radicals Determination in "In Vitro" Biological [illegible]

Spin adduct of the 1HEt radical was detected by the method described previously [18,20]. Briefly, in experiments involving cytosolic activation of ethanol, selected ion monitoring (SIM) of mass spectrum of the adduct was employed to increase sensitivity. Selected masses were 250 (M-·$CHCH_3OTMS$) and 194 (m/z 250-C_4H_8). Purified cytosolic fractions (12.3 ± 0.8 mg cytosolic protein per ml) were added to 25 mM PBN, 0.25 mM hypoxanthine, and 0.28 M ethanol. In the case of experiments with DMSO (0.3 M), PBN was 9.4 mM because of the lower solubility of the spin trap in the absence of any solvent. All other components in incubation were at the same concentration as described above. 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:acetonitrile (1:1), 60°C, 15 min, and analyzed by GC/MS-SIM. Chromatographic conditions were at follows: column, 5% phenylmethyl silicone, 12 m × 0.2 mm i.d., programmed from 100°C to 300°C at a ramp of 10°C/min. Injection port was at 250°C and transfer line to MS, 300°C. Dwell time was 50 ms for both masses selected.

RESULTS

Ethanol Biotransformation to Acetaldehyde in Cytosolic Fraction

Results on acetaldehyde levels in incubation mixtures containing the cytosolic fraction of ventral prostate are summarized in Table I. The reaction was sensitive to heating (5 min at 100°C), not enhanced by NAD^+ and not inhibited by pyrazole.

The ethanol metabolism to acetaldehyde was significantly enhanced by NADH, hypoxanthine, and xanthine and those increases were inhibited by allopurinol but not by desferrioxamine (DFA). Methyl xanthines like caffeine, theobromine, theophylline, or 1,7-dimethylxanthine were also able to induce the generation of acetaldehyde when present in the place of hypoxanthine. That process was also inhibited by allopurinol. Replacement of purines by N-methylnicotinamide did not enhance significantly the ability of prostate cytosol to biotransform ethanol to acetaldehyde.

1-Hydroxyethyl Radicals Detection in In Vitro Experiments With Ventral Prostate Cytosolic Fraction

Figure 1 shows the capillary GC analysis with SIM detection of reaction products arising from ethanol biotransformation by ventral prostate cytosolic fraction in the presence of the spin trap PBN. Spin adduct of the 1HEt radical was detected only

TABLE 1. Ethanol Biotransformation to Acetaldehyde by Ventral Prostate Cytosol

Experimental[a]	Acetaldehyde (ng)/protein (mg)
Control (no PMSF)[b]	14.57 ± 0.85
Control[c]	14.09 ± 0.30
0.3 mM NAD^+ (no PMSF)[b,d]	16.95 ± 1.35
0.3 mM NAD^+[e]	18.32 ± 0.50
NAD^+ +5 mM pyrazole[f]	16.35 ± 0.89
NAD^+ + allopurinol	0.43 ± 0.04
Heated (100°C, 5 min)	1.03 ± 0.14
Heated + hypoxanthine	1.05 ± 0.06
Allopurinol	1:06 ± 0.19
Hypoxanthine	36.92 ± 2.12
Hypoxanthine + allopurinol	0.77 ± 0.08
Hypoxanthine + NAD^+	28.44 ± 0.63
Hypoxanthine + NAD^+ + allopurinol	0.45 ± 0.05
Xanthine	28.34 ± 1.20
Xanthine + allopurinol	0.97 ± 0.10
Caffeine	23.20 ± 0.44
Caffeine + allopurinol	0.79 ± 0.10
Theobromine	21.63 ± 1.09
Theobromine + allopurinol	0.78 ± 0.04
Theophylline	19.52 ± 0.56
Theophylline + allopurinol	0.80 ± 0.17
1,7-dimethylxanthine	22.68 ± 0.43
1,7-dimethylxanthine + allopurinol	0.78 ± 0.13
0.3 mM NADH	25.41 ± 1.74
0.3 mM NADH + allopurinol	0.48 ± 0.03
Hypoxanthine + 25 mM sodium benzoate[g]	31.46 ± 0.72
Hypoxanthine + 1 mM DFA[h]	36.61 ± 2.24
2.5 mM N-methylnicothinamide	16.65 ± 0.64
N-methylnicothinamide + 10 μM menadione	16.25 ± 0.70

[a]Incubation mixtures containing cytosol (12.4 ± 3.0 mg of cytosolic protein/ml), 0.21 M ethanol and, when indicated, 0.25 mM hypoxanthine (xanthine, caffeine, theobromine, 1,7-dimethylxanthine, or theophylline), 0.15 mM allopurinol, were conducted for 1 h at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 ml NaCl saturated solution. See Methods for other details. Each result is the mean of three separate samples.

[b]In this group cytosol prepared in the absence of phenylmethylsulfonyl fluoride (PMSF) was used for the experiment. Otherwise, cytosolic fraction was prepared in the presence of 1 mM PMSF, to prevent proteolysis of XDh to give XO.

[c]$P > 0.05$ when compared to control (no PMSF).

[d]$P > 0.05$ when comapred to control (no PMSF).

[e]$P > 0.05$ when compared to control.

[f]$P > 0.05$ when compared to (0.3 mM NAD) group.

[g]$P < 0.05$ when compared to hypoxanthine.

[h]$P > 0.05$ when compared to hypoxanthine.

when hypoxanthine or xanthine were present (traces of PBN-1HEt were observed in control samples). Cytosolic fraction was very efficient to produce 1HEt from ethanol (Fig. 1a–d). The process was significantly inhibited by desferrioxamine. Methylxanthines were able to promote 1HEt formation via the xanthine oxidase pathway but in a lower extent than hypoxanthine (Fig. 2a–d).

In addition, two more peaks due possibly to the interaction between ·OH radicals and PBN were observed (peaks A and B) but not the adduct corresponding to

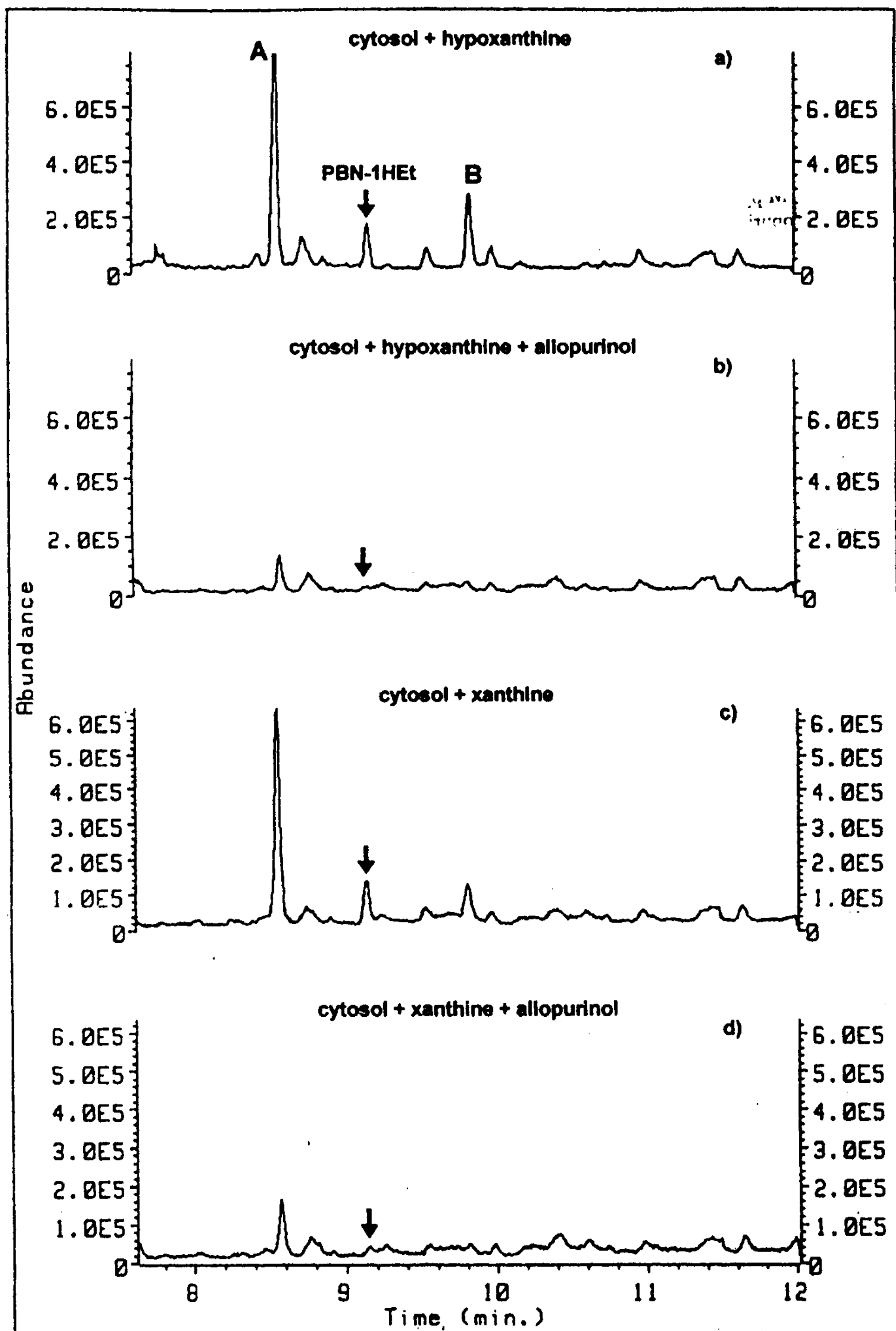

Fig. 1. Selected-ion current profile obtained from GC-MS-SIM analysis of a sample from incubation containing cytosol, and 0.21 M ethanol in the presence of 25 mM N-t-butyl-α-phenylnitrone (PBN), after trimethylsilylation. See Method for other details. **a:** With 0.25 mM hypoxanthine. Peaks: A and B, hydroxyl derived PBN adducts; PBN-1HEt, 1-hydroxyethyl-PBN adduct. **b:** 0.25 mM hypoxanthine in the presence of 0.15 mM allopurinol. **c:** 0.25 mM xanthine. **d:** 0.25 mM xanthine + 0.15 mM allopurinol.

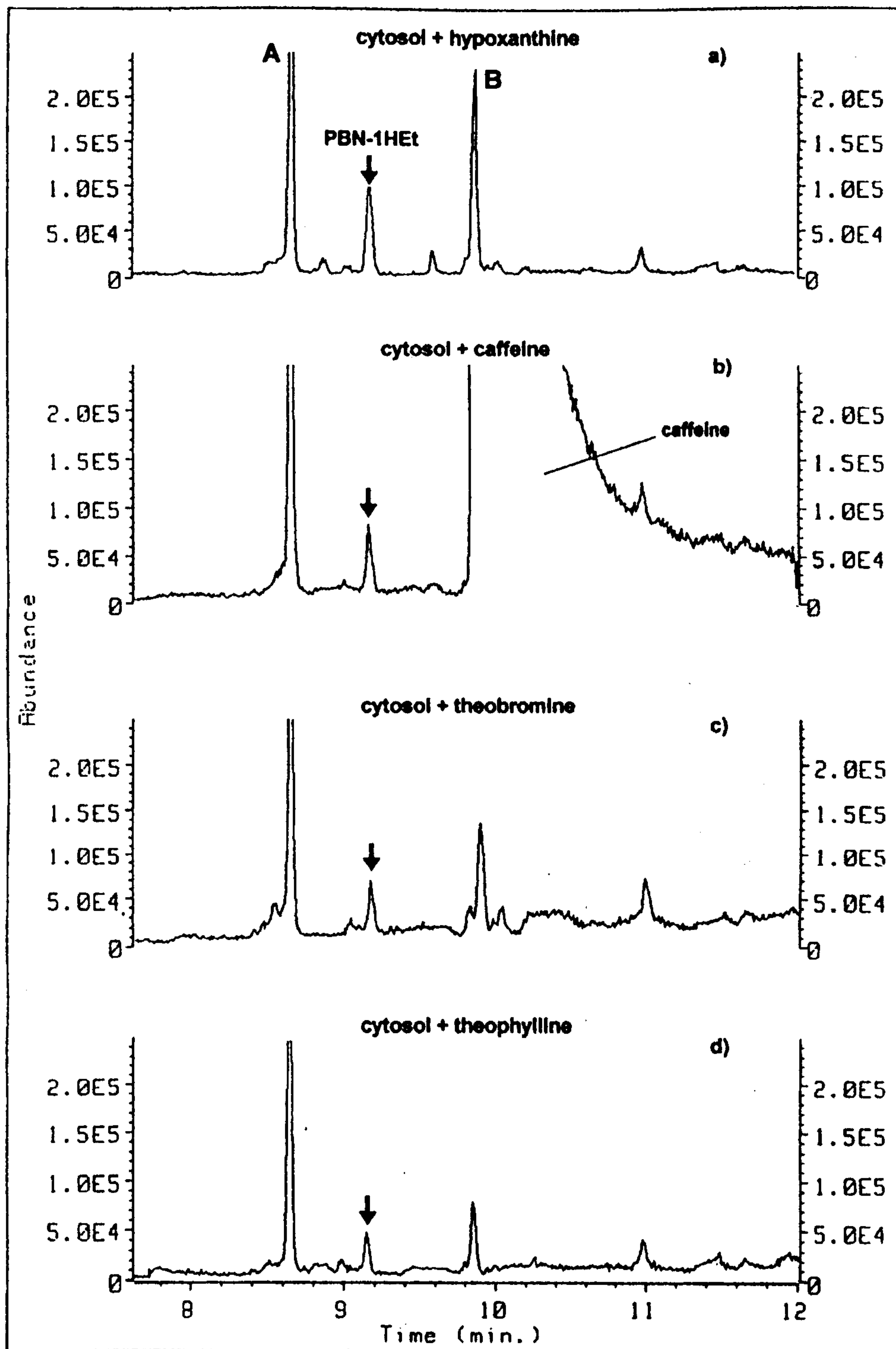

Fig. 2. Comparative effect of methylxanthines on the xanthine oxidase activity and the formation of 1-hydroxyethyl radicals. **a:** 0.25 mM hypoxanthine. **b:** 0.25 mM caffeine. **c:** 0.25 mM theobromine. **d:** 0.25 mM theophylline.

the addition of the hydroxyl radical to the nitrone. These compounds were reported previously by us to be formed in the liver microsomal biotransformation of ethanol [21]. Hydroxyl addition to the aromatic ring of the spin trap can give account for the proposed structures in the case of compounds A and B. Specific place of linkage of the hydroxyl group in the aromatic ring could not be deduced from mass spectra. Their formation was observed also in the absence of ethanol and was inhibited by desferrioxamine.

The involvment of free radical species in the formation of these peaks was evidenced by the quenching effect by DMSO (Fig. 3a–d).

DISCUSION AND CONCLUSIONS

The obtained results show that the rat ventral prostate cytosolic fraction, in the presence of different XO substrates, is able to promote the oxidation of ethanol to acetaldehyde and 1HEt. The substrates tested were NADH, hypoxanthine, xanthine, caffeine, and other methylated xanthines [22,23]. In additional support to the XO participation in this process is the fact that the oxidation of alcohol to the acetaldehyde and 1HEt was inhibited by allopurinol, a very well-known inhibitor of XO activity [22,23].

Furthermore, allopurinol also inhibited the ability of the control homogenate (in the absence of added XO substrates) to catalyze the oxidation of EtOH to acetaldehyde, evidencing that the homogenate itself contains XO substrates of endogenous nature (e.g., NADH, xanthine, hypoxanthine, or other). The fact that there was not an enhancing effect by NAD^+ on acetaldehyde production would indicate that xanthine dehydrogenase (XDh) contribution was not significant and that alcohol dehydrogenase (ADh) was not involved [24]. The lack of pyrazole effect in this process reinforces that hypothesis [24]. The lack of enhancing effect by N-methylnicotinamide also excludes the participation of the other molybdo enzyme, aldehyde oxidase [22,23].

The production of 1HEt radicals under similar experimental conditions implies that during these processes ·OH radicals should be formed. The mechanisms of hydroxyl radicals formation was postulated to be involved in the case of liver XO generation of superoxide radicals, their dismutaton to H_2O_2 and the iron-mediated production of ·OH [25].

The present studies are in agreement with the possibility that ·OH radicals were operating for the case of prostate cytosol and support iron participation in that process. In effect, the bioactivation of ethanol to 1HEt is significantly inhibited by DFA, a very potent and specific iron chelator [26,27]. Free iron is not regularly available under in vivo conditions, and that could cast some doubts about the possibility of these reactions to occur under in vivo conditions. Notwithstanding, it is also known that under oxidative stress conditions labile forms of iron might be available and catalyze these type of reactions [25,28]. Further, benzoate, a ·OH radical reactive chemical [29], was able to significantly decrease the formation of acetaldehyde. Also, the production of 1HEt is fully inhibited by DMSO, a known ·OH radical trapping agent [30].

Unfortunately, in that experiment it was not possible to detect the methyl adduct of PBN (resulting from methyl radical formation during ·OH attack to DMSO) due to lack of sensitivity of our MS system. That decrease in sensitivity might result from competition between DMSO and EtOH to scavenge the ·OH radicals and the

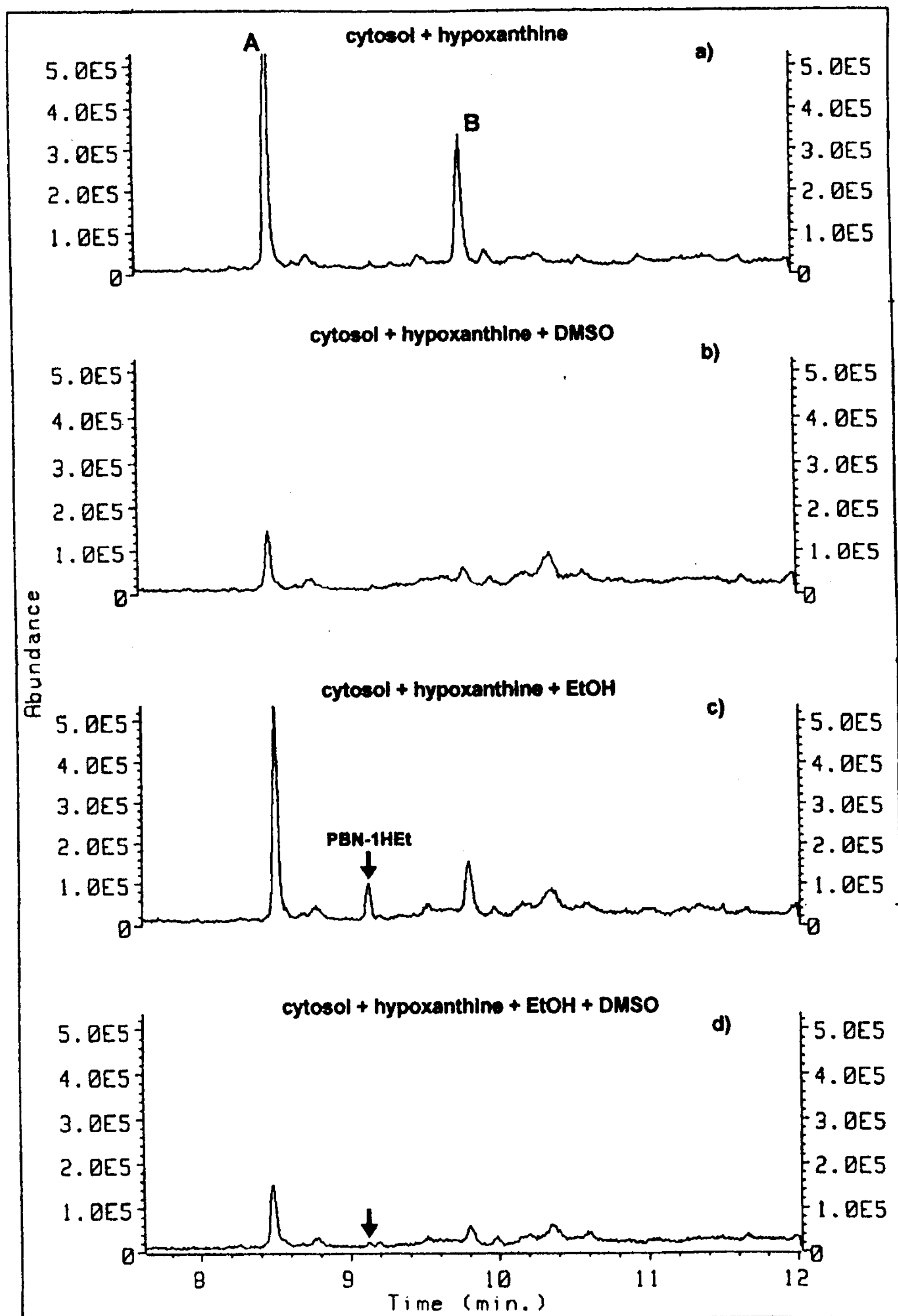

Fig. 3 Selected-ion current profile obtained from GC-MS-SIM analysis of a sample containing cytosol and 9.4 mM PBN, after trimethylsilylation. **a:** 0.25 mM hypoxanthine. **b:** the same as in a) plus 0.3 M dimethyl sulfoxide (DMSO). **c:** 0.25 mM hypoxanthine plus 0.28 M ethanol (EtOH); **d** is the same as in c) plus 0.3 M DMSO. See Method for other details.

competition of the pathway leading to methane [30]. In contrast, the observed results on acetaldehyde production by XO are not attributable to iron-mediated oxidations of ethanol, since the process is not inhibited by DFA. However, they do not allow at present to completely understand how the process occurs. That remains to be explained.

In summary, as result of the prostatic XO assisted biotransformation of EtOH several different bioactive moieties might be formed. They include acetaldehyde, reactive oxygen species like superoxide anion radical, hydrogen peroxide, and hydroxyl radical and a carbon-centered radical, the 1-hydroxyethyl.

Several biological consequences might be expected from their formation and from the subsequent oxidative stress resulting thereof. In effect, free radicals and oxidative stress has been proposed to play roles in both the initiation and the promotion stages of ethanol carcinogenesis [14,15], in cell signaling for proliferation [31–33], in chemically induced cell injury and in apoptosis [28,34–36].

The other product formed during ethanol biotransformation, acetaldehyde, is also a reactive molecule able to interact with DNA bases, proteins, and phosphatidylethanolamine, to deplete GSH and generate an acetyl radical [24]. Furthermore, acetaldehyde was reported to be mutagenic and carcinogenic [12,13,37].

These studies and other preliminary work from our laboratory on additional sources of acetaldehyde and free radicals formation, during ethanol biotransformation in other prostate organelles [38], might provide the biological mechanistic support requested in recent reviews on this matter [3,4] to the possibility that heavy alcohol consumption played some role in prostate cancer induction in humans [5–11]. In addition, the present results might suggest the possibility that simultaneous factors might play some cooperative or synergistic effects with that of ethanol to favor occurrence of prostate cancer. One of them might be related to the high consumption of purines-rich food (e.g., meat).

Some of the here-tested cosubstrates of XO promoting ethanol biotransformation to acetaldehyde and 1HEt (e.g., hypoxanthine or xanthine) are known products of nucleoprotein degradation [39]. Furthermore, alcohol consumption itself also promotes purines degradation and increases uric acid formation [40–43]. High meat consumption is known to be by itself a relevant factor in prostate cancer promotion [44].

Another potential contributing factor to ethanol promotion of prostate cancer in alcoholics suggested by present studies might be the high consumption of caffeine and/or methylxanthines-rich containing drinks and beverages in conduction with that of alcohol. In effect, the synergistic cosubstrates tested in the present studies as required for XO-mediated ethanol biotransformation to acetaldehyde and 1HEt are either present in those drinks and beverages or they are metabolites formed from them in the body [45, 46]. Notwithstanding, no correlation was reported to exist between coffee drinking as only factor and prostate cancer [1,45].

The present studies might suggest the convenience of considering epidemiological studies where multifactorial causes are carefully weighed as contributing factors to ethanol effects on prostate cancer.

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