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Rat ventral prostate xanthine oxidase–mediated metabolism of acetaldehyde to acetyl radical

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Alcohol drinking is known to lead to deleterious effects on prostate epithelial cells from humans and experimental animals. The understanding of the mechanisms underlying these effects is relevant to intraprostatic ethanol treatment of benign prostatic hyperplasia and to shed some light into the conflictive results linking alcohol consumption to prostate cancer. In previous studies, we provided evidence about the presence in the rat ventral prostate of cytosolic and microsomal metabolic pathways of ethanol to acetaldehyde and 1-hydroxyethyl radical and about the low levels of alcohol dehydrogenase and aldehyde dehydrogenase. Acetaldehyde accumulation in prostate tissue and oxidative stress promotion were also observed. In this study, we report that in the ventral prostate cytosolic fraction, xanthine oxidoreductase is able to metabolize acetaldehyde to acetyl radical. The identification of the acetyl was performed by GC-MS of the silylated acetyl-PBN adduct. Reference adduct was generated chemically. Formation of acetyl was also observed using pure xanthine oxidase. The generation of acetyl by the prostate cytosol was inhibited by allopurinol, oxypurinol, diphenyleneiodonium chloride, folate, and ellagic acid. Results suggest that metabolism of ethanol to acetaldehyde and to 1-hydroxyethyl and acetyl radicals could be involved in the deleterious effects of alcohol drinking on prostate epithelial cells.

Key words: acetaldehyde; acetyl; alcohol; free radicals; prostate; xanthine oxidase

Introduction

In the course of previous studies from others and from our laboratory, evidence was provided about deleterious effects of repetitive alcohol drinking on prostate epithelial cells from humans and experimental animals.[1–7] Those results were considered of relevance in relation both to the intraprostatic treatment with pure alcohol of urinary retention in men with benign prostatic hyperplasia and to the conflictive results linking alcohol consumption with prostate cancer. To attempt to shed some light into these matters, our laboratory studied the potential metabolic activation of alcohol to deleterious metabolites by rat ventral prostate tissue. In an initial work, we reported that the cytosolic fraction can metabolize ethanol to acetaldehyde and to 1-hydroxyethyl radicals in a process mediated by xanthine oxidoreductase (XOR).[8] Later studies evidenced the existence of an additional pathway of metabolic activation of ethanol to acetaldehyde and 1-hydroxyethyl at the prostate microsomal fraction. This process involved NADPH, oxygen, and cytochrome P450 in part of it.[9] Recent preliminary studies reported the presence of other CYP2E1-mediated enzymatic activities in the rat ventral prostate microsomes and their induction by repetitive ethanol drinking.[10] Interestingly, those studies also did mention to the presence of a poor to null activity in prostate tissue of alcohol dehydrogenase (ADh) and, more important, of aldehyde dehydrogenase (AldDh). The latter was apparently responsible for a long period of acetaldehyde accumulation in prostate tissue after ethanol drinking. Further, evidence of an oxidative-stress response of prostate tissue after repetitive alcohol drinking was also anticipated.[10] In this study, we report that any acetaldehyde present in prostate tissue and known to accumulate to some extent there might be a source of additional free radicals of acetyl nature.

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Materials and methods

Chemicals

Acetaldehyde (analytical grade) was from Fluka (Buchs, Switzerland). Perdeuterated acetaldehyde (ethanal-d_4) was from Aldrich (Milwaukee,

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Wisconsin, USA). N-*t*-butyl-α-phenylnitrone (PBN), XOR (grade III, from butter milk, EC 1.17.3.2), and the drugs tested on their effects on the metabolism of acetaldehyde (hypoxanthine, diphenyleneiodonium chloride (DPI), folic acid, ellagic acid, oxypurinol, allopurinol) were from Sigma Co. (St. Louis, Missouri, USA). Nitrogen (ultrahigh purity) was from AGA (San Martin, Argentina) and was further deoxygenated by bubbling through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1N NaOH.

Animals and treatments

Noninbred 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*. 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). Animals were killed by decapitation and their ventral prostates were rapidly excised and processed. Purified cytosolic fractions were obtained as previously described and were essentially free from cross contamination; 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the homogenizing buffer to avoid the irreversible conversion (by proteolisis) of xanthine dehydrogenase (XDh) to xanthine oxidase (XO).[8] We did not use dithioerythritol in the cytosol preparation procedure because in that case, conversion to the XDh form is nearly quantitative. Cytosol was not further dyalized except when indicated for some of the experiments. In this case, supernatant fraction obtained from centrifugation at 105,000 × *g* was dialyzed overnight in the cold room against 2 L of homogenizing buffer containing 1 mM PMSF.[8]

Acetyl radical adduct generation in chemical model systems

Spin adduct of acetyl radical was generated in purely chemical reaction systems to obtain an amount high enough to allow its structural characterization by mass spectrometry and to select appropriate masses for selected ion monitoring (SIM) detection, necessary in the case of real biological samples. Two model systems were used for this purpose.[11]

Fenton reaction A "Fenton system" was prepared essentially as follows: 5 mM ferrous sulfate (prepared freshly in nitrogen-purged water), 5 mM hydrogen peroxide, 0.2 M acetaldehyde, and 11 mM PBN in 50 mM phosphate buffer, pH 7.4. Blank samples without acetaldehyde or without H_2O_2 were run simultaneously. After addition of ferrous sulfate, the reaction was immediately extracted with 500 μL isooctane, the organic phase was separated and evaporated under nitrogen, and then silylated with a mixture of BSTFA:acetonitrile (1:1) and analyzed by GC-MS. Total ion current chromatographic analysis and mass spectrometric identification of reaction products were performed in a Hewlett Packard model 5970B mass-selective detector interfaced to a HP 5890 gas chromatograph. Chromatographic conditions were as follows: column, methyl silicone, 12 m × 0.2 mm i.d. (0.33 μm film thickness), programmed from 100 °C (1 min) to 150 °C at a ramp of 10 °C/min and then to 190 °C at 4 °C/min. Injection port was at 250 °C in the splitless injection mode. Transfer line to MS was at 280 °C. Spectra were taken at 70 eV scanning quadrupole from 40 to 500 amu. Solvent delay was 7.3 min.

Acetyl radical generation in the FAD–NADPH model system To evaluate the ability of the redox system constituted by FAD and NADPH to generate free radicals from acetaldehyde, the spin adduct of the acetyl radical was generated in this chemical system, essentially as previously described by our laboratory.[11] In this case, incubation mixture contained 1 mM FAD, 9.4 mM PBN, 0.12 M acetaldehyde, and NADPH generating system (0.45 mM $NADP^+$, 4 mM dl-isocitric acid trisodium salt, and 0.25 U of isocitric dehydrogenase) in 50 mM K_2HPO_4 buffer, pH 7.4. The reaction mixture was incubated for 1 h at 37 °C under nitrogen. Control samples performed in the absence of acetaldehyde, FAD, or NADPH generating system were run simultaneously. The reaction volume was extracted with 500 μL isooctane, the organic phase separated and evaporated under N_2, and then silylated with a mixture of BSTFA:acetonitrile (1:2) at 60 °C for 15 min. GC-MS conditions remain the same as mentioned above.

In both model systems, fragmentation patterns in spectra were confirmed by using ethanal-d_4, the perdeuterated analog, and looking for the mass shifts.

Biotransformation of acetaldehyde to acetyl radical by pure XO

The incubation mixture contained XO grade III (0.317 units), 9.4 mM PBN, 0.12 M acetaldehyde in 50 mM K_2HPO_4 buffer, pH 7.4. When indicated, hypoxanthine of 0.25 mM or allopurinol of 0.15 mM was added to the mixture. Control samples without XO or acetaldehyde were run simultaneously. After incubation for 1 h at 37 °C, the reac-

tion volume (3 mL) was extracted with 500 µL isooctane, centrifuged, and the organic layer was evaporated under nitrogen. The residue was silylated with BSTFA:acetonitrile (1:1) and analyzed by GC-MS. Samples were analyzed in the SIM mode of detection to increase sensitivity. Selected masses were 250 ($M^{\bullet+}$ – $COCH_3$) and 194 (m/z 250 – C_4 H_8). Dwell time was 50 ms for both masses.

Acetyl radical detection in in-vitro experiments with ventral prostate cytosolic fraction

Spin adduct of the acetyl radical was detected by the method developed in our laboratory for the detection of 1-hydroxyethyl radicals.[12] Briefly, in experiments involving cytosolic activation of acetaldehyde, SIM of mass spectrum of the adduct was used to increase sensitivity (selected masses as above). Incubation mixtures containing purified nondialyzed cytosol (11.1 ± 1.5 mg cytosolic protein per mL) in sucrose-tris-potassium-magnesium (STKM) buffer (0.25 M sucrose/50 mM Tris-HCl, pH 7.5/2.5 mM KCl/5 mM $MgCl_2$) were added to 9.4 mM PBN and 0.12 M acetaldehyde (3 mL final volume). Several compounds were tested for their effect on the formation of acetyl: 0.25 mM hypoxanthine, 0.15 mM allopurinol, 0.15 mM oxypurinol, 10 µM DPI, 10 µM folic acid, and 10 µM ellagic acid. Incubations were performed in aluminum-sealed neoprene-septum stoppered glass vials and conducted for 1 h at 37 °C under air atmosphere. Reaction was interrupted by placing in the cold. The volume (3 mL) was extracted with 500 µL isooctane and the extract was processed as described above.

Results

Acetyl radical detection in chemical reaction model systems

Acetaldehyde reacted with hydroxyl radicals in the Fenton system to generate acetyl radicals. Spectrum showed in Figure 1A corresponded to the adduct formed between the spin trap and the acetyl. The molecular mass was assigned to m/z 293 and confirmed by m/z 278 ($M^{\bullet+}$ –15) and m/z 250 ($M^{\bullet+}$ minus the acetyl radical). Other important fragments were m/z 222 (loss of isobutylene from m/z 278), m/z 194 (loss of isobutylene from m/z 250), m/z 104 ($C_6H_5CNH^+$), and m/z 43 (acetyl cation). Fragmentation pattern was confirmed by running a sample containing the perdeuterated acetaldehyde (ethanal-d_4) and looking for the mass shifts (see Figure 1B). When acetaldehyde was tested in the FAD–NADPH model system, same peak was observed.

Acetyl generated from acetaldehyde by pure XO from butter milk

Pure XO was able to oxidize acetaldehyde to acetyl, either in the presence or in the absence of hypoxanthine as cosubstrate (Figures 2A and 2C). However, in the absence of the purine, the intensity of the adduct was lower, as reflected by the voltage needed for detection. In both cases, allopurinol was able to deplete the peak intensity for the PBN-acetyl adduct (Figures 2B and 2D). Two additional peaks were detected in this experimental system. One of them corresponded to an aromatic ring hydroxylated PBN adduct, resulting from the interaction between hydroxyl radical and PBN, as described previously by us under other experimental conditions.[12,13] This peak was also depleted by allopurinol. The other minor peak was not identified as any of those previously described by us (e.g., another isomer of the

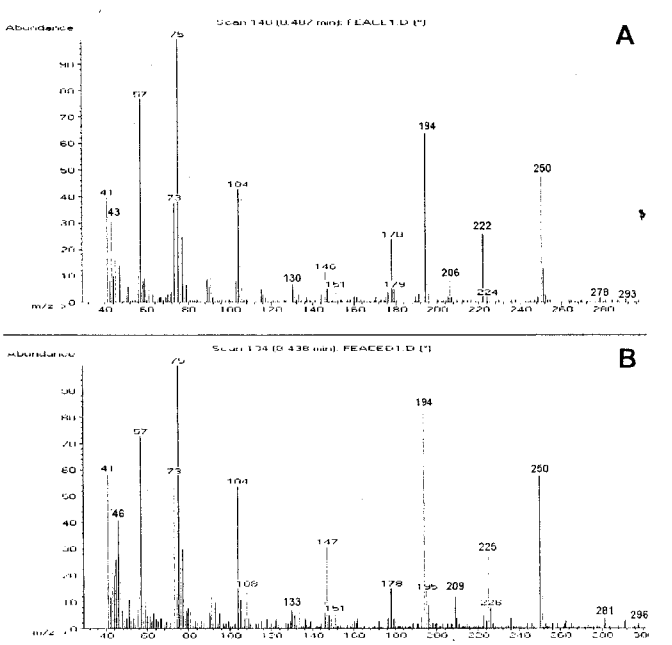

Figure 1 (A) Mass spectrum taken from the PBN adduct of acetyl (PBN-Ac). The molecular mass was assigned to m/z 293 and confirmed by m/z 278 ($M^{\bullet+}$ –15) and m/z 250 ($M^{\bullet+}$ minus the acetyl radical). Other important fragments were m/z 222 (loss of isobutylene from m/z 278), m/z 194 (loss of isobutylene from m/z 250), m/z 104 ($C_6H_5CNH^+$), and m/z 43 (acetyl cation). (B) Mass spectrum taken from the PBN adduct of acetyl (PBN-Ac) when using the perdeuterated analog of acetaldehyde (ethanal-d_4). Molecular mass was assigned to m/z 296 and confirmed by m/z 281 ($M^{\bullet+}$ –15) and m/z 250 ($M^{\bullet+}$ minus the acetyl radical). Other important fragments were m/z 225 (loss of isobutylene from m/z 278), m/z 194 (loss of isobutylene from m/z 250), m/z 104 ($C_6H_5CNH^+$), and m/z 46 (acetyl cation).

hydroxylated PBN or the 1-hydroxyethyl-PBN adduct). Its presence could be showed only when samples were run in the SIM mode if m/z 250 and m/z 194 were selected. This should be interpreted as suggesting the presence of other PBN adduct since the m/z 250 and m/z 194 are fragments representing the core of the PBN structure. We were unable to decipher the precise of this adduct because when running of the samples was made in the scan mode, the unknown peak was not detected and that prevented to obtain the full spectrum necessary for identification. The SIM mode being more sensitive than the scan mode made possible its detection. In addition, the absence of the unknown peak in the chromatograms derived from studies in model systems further prevented to obtain the necessary spectral information.

Acetyl generated from acetaldehyde by rat ventral prostate cytosol

Nondialyzed rat prostate cytosol was able to oxidize acetaldehyde to acetyl radicals (Figure 3A). When using dialyzed cytosol in incubations, same result was obtained. Acetyl formation was not dependent on the presence of hypoxanthine either (data not shown) and was inhibited by allopurinol and oxypurinol (Figures 3B and 3C, respectively). Allopurinol has been widely considered a specific inhibitor of XO and a drug used in gout treatment (where the XO-mediated production of uric acid from purines is a key factor). Oxypurinol is the actual metabolite inactivating the enzyme after oxidation of allopurinol by XO. Diphenyleneiodonium, a known inhibitor agent for flavin containing enzymes, lead to a complete inhibition of acetyl formation (Figure 3D).[14] Two other compounds known by their ability to inhibit the enzyme, folic acid, and ellagic acid were also effective to deplete or suppress the formation of acetyl (Figures 3E and 3F).[15] In effect, we observed that in mammary tissue, both ellagic acid and folic acid suppressed the generation of acetaldehyde up to 98% in a biotransformation mediated by XOR activity.[15] An unknown peak was detected at the same retention time than for the one detected in experiments performed with the pure enzyme. This peak was not equally sensitive to the effect of the XOR inhibitors tested than the PBN-Ac. The difference observed in the behavior of the different XO inhibitors tested in relation to the formation of the unknown peak cannot be explained at present considering the lack of knowledge about its structure.

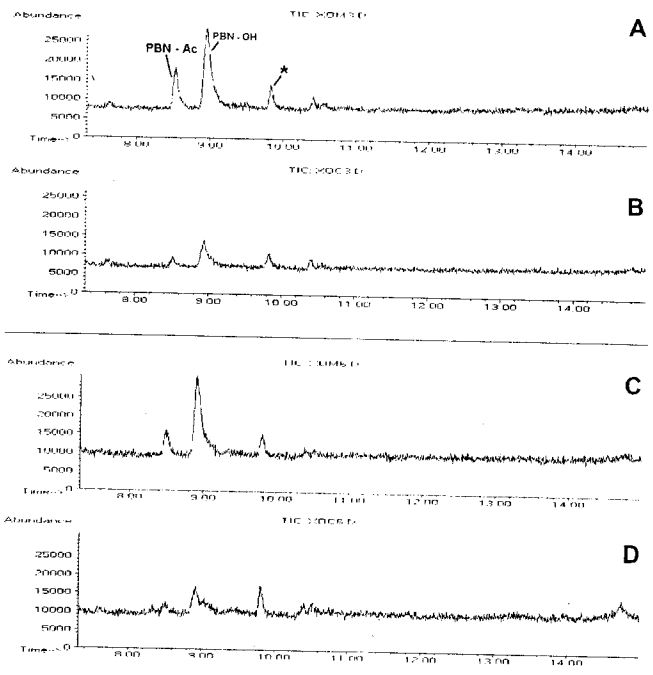

Figure 2 Selected ion current profile obtained from GC-MS-SIM analysis of a sample of incubation containing pure xanthine oxidase, acetaldehyde, and PBN after trimethylsilylation. Selected masses were 250 ($M^{\bullet+}$ – $COCH_3$) and 194 (m/z 250 – C_4 H_8). Dwell time was 50 ms for both masses. (A) In the presence of 0.25 mM hypoxanthine. Electron multiplier was set at 2400 V for detection. PBN-Ac, PBN-acetyl spin adduct; PBN-OH, hydroxylated PBN; *, unknown. (B) The same as in (A) but in the presence of 0.15 mM allopurinol. (C) The same as in (A) but in the absence of hypoxanthine. Electron multiplier was set at 2600 V for detection. (D) The same as in (C) but in the presence of 0.15 mM allopurinol.

Discussion

In the course of previous studies from our laboratory, we reported that the rat ventral prostate cytosol and the microsomal fraction were able to metabolize alcohol to acetaldehyde and to 1-hydroxyethyl radical.[8,9] More recently, our laboratory showed that rat ventral prostate microsomes exhibited some CYP2E1-mediated metabolic activity that was inducible by repetitive ethanol drinking.[10] However, rat prostate tissue evidenced to have very low ADh and AldDh activities.[10] Further, as a result of the own capacity to generate acetaldehyde and of the lack of ability to handle acetaldehyde either produced *in situ* or arriving from the liver or other tissues via blood, a significant accumulation of the aldehyde was observable.[10] The exposure of rat prostate tissue to repetitive ethanol drinking condition also led to oxidative stress production and to deleterious ultrastructural effects.[2,10] The oxidative stress observed was attributed in those studies to 1-hydroxyethyl production and to decreased antioxidant defenses present in prostate after repetitive ethanol drinking. In this study, the potential contribution of an additional

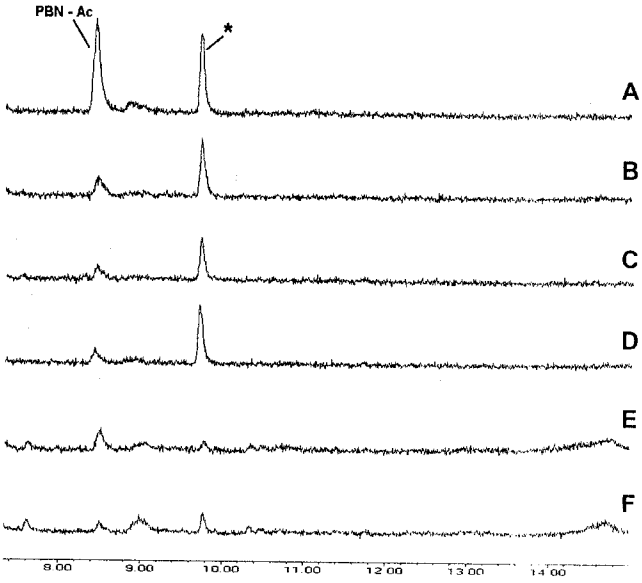

Figure 3 (A) Selected ion current profile obtained from GC-MS-SIM analysis of a sample of incubation containing nondialyzed rat ventral prostate cytosol, acetaldehyde, and PBN after trimethylsilylation. Masses selected were 250 and 194 and electron multiplier was set at 2400 V for detection. PBN-Ac, PBN-acetyl spin adduct; *, unknown. (B) The same as in (A) but in the presence of 0.15 mM allopurinol. (C) The same as in (A) but in the presence of 0.15 mM oxypurinol. (D) The same as in (A) but in the presence of 10 μM DPI. (E) The same as in (A) but in the presence of 10 μM folic acid. (F) The same as in (A) but in the presence of 10 μM ellagic acid.

source of free radicals to the oxidative stress is reported. In effect, we found that the prostate cytosolic fraction was able to activate acetaldehyde to acetyl. We identified its formation via GC-MS of the PBN spin adduct, conveniently derivatized in a process similar to the one used for hydroxyl, 1-hydroxyethyl, or hydroxymethyl radicals under different experimental conditions.[12,13,16,17] It is important to note, however, that radical detection by this GC-MS methodology is only semiquantitative. This is because it is not possible to have a stable reference compound (the PBN-acetyl), allowing the precise quantitative determination of the adduct formed in each case. Other established techniques to measure the formation of free radicals, as the electron spin resonance, also suffers the same limitation. The reference acetyl radicals for comparing mass spectra and for selecting ions for GC-MS-SIM were generated by two different methods, Fenton reaction and FAD semiquinone activation system previously reported by our laboratory.[11] The occurrence of the latter bioactivation process proved to be of special interest when attempt to identify the nature of the enzymatic process involved in the cytosolic metabolism of acetaldehyde to acetyl radical. In effect, the participating enzyme was strongly inhibited by low concentrations of DPI. This compound is a known specific inhibitor of flavin-mediated enzymatic metabolic transformations.[14] The additional inhibitory response to allopurinol, oxypurinol, and folic acid suggests that XOR might be involved in the process. Allopurinol and oxypurinol are well-established inhibitors of XOR, and folic acid also inhibit this enzyme at low concentration.[15,18] Ellagic acid is a polyphenol antioxidant found in many fruits and vegetables, including raspberries, strawberries, cranberries, walnuts, pecans, pomegranates, and other plant foods, and in the case of mammary tissue also proved to inhibit XOR-mediated acetaldehyde production up to 95%.[15] XOR is a flavoenzyme present in low amounts in prostate cytosol.[8] Our results on the activation of acetaldehyde to acetyl radical by FAD semiquinone might indicate that FAD-containing site of the enzyme is involved in the activation process. The fact that some activation of acetaldehyde by XO can be observed even in the absence of the cosubstrate could be interpreted as indicating that part of the FAD moiety in XO is already in the semiquinone form. The presence of cosubstrate would enhance the efficiency of that semiquinone formation. Alternatively, a nondialyzable molecule able to switch FAD to its semiquinone in the cytosol should be present. Dialysis of cytosol does not prevent the activation of acetaldehyde. Both alternative hypotheses require the involvement of the FAD site of the enzyme as evidenced by their susceptibility of the process to DPI. However that remains to be proved.

The contributions of the previously evidenced metabolic pathways of ethanol oxidation and the results shown here to prostate epithelial cell injury or to the conflictive epidemiological observations linking ethanol consumption to prostate cancer are still far from clear.[1,2]

The present and the previous results from our laboratory strongly suggest that during the intraprostatic ethanol treatment of benign prostatic hyperplasia, this tissue would be exposed to significant concentrations of both the alcohol and acetaldehyde.[8,9] This is because, at least in the rat, ventral prostate showed to efficiently oxidize ethanol to both acetaldehyde and 1-hydroxyethyl radicals via microsomal and cytosolic pathways, and due to the limited activity of AldDh present, acetaldehyde accumulates.[10] In the case of the cytosolic metabolism, the role of XO was proved by the inhibition with allopurinol and by its stimulation with a variety of purines.[8] In the case of the intraprostatic ethanol treatment, it is tempting to speculate that as a result of the acetaldehyde generated and accumulated in this tissue and because of the free radicals produced *in situ*, the cell injury

needed to obtain the chemotherapeutic effect may result.

There is, however, an interesting point to remark. It concerns the potent inhibitory effects of folic acid on acetaldehyde activation to acetyl reported here. In effect, in previous epidemiological studies by Pelucchi, *et al.* the authors reported that protection conferred on prostate cancer risk by dietary folate was stronger in high alcohol drinkers.[6] Further, they also mention that the combined effect of high folate and low alcohol intake further decreased prostate cancer risk up to 54% risk reduction. Whether these epidemiological observations are linked or not to our previous and present results on alcohol or acetaldehyde activation to 1-hydroxyethyl and acetyl radicals by XO and their inhibition by folic acid or their derivatives is not known at present but could be an alternative to other postulated possibilities considered by those workers.[6,15]

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