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Cytosolic xanthine oxidoreductase mediated bioactivation of ethanol to acetaldehyde and free radicals in rat breast tissue. Its potential role in alcohol-promoted mammary cancer

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Abstract

Epidemiological evidence links alcohol intake with increased risk in breast cancer. Not all the characteristics of the correlation can be explained in terms of changes in hormonal factors. In this work, we explore the possibility that alcohol were activated to acetaldehyde and free radicals in situ by xanthine dehydrogenase (XDh) and xanthine oxidase (XO) and/or aldehyde oxidase (AO). Incubation of cytosolic fraction with xanthine oxidoreductase (XDh + XO) (XOR) cosubstrates (e.g. NAD^+, hypoxanthine, xanthine, caffeine, theobromine, theophylline or 1,7-dimethylxanthine) significantly enhanced the biotransformation of ethanol to acetaldehyde. The process was inhibited by allopurinol and not by pyrazole or benzoate or desferrioxamine and was not accompanied by detectable formation of 1HEt. However, hydroxylated aromatic derivatives of PBN were detected, suggesting either that hydroxyl free radicals might be formed or that XOR might catalyze aromatic hydroxylation of PBN. No bioactivation of ethanol to acetaldehyde was detectable when a cosubstrate of AO such as *N*-methylnicotinamide was included in cytosolic incubation mixtures. Results suggest that bioactivation of ethanol in situ to a carcinogen, such as acetaldehyde, and potentially to free radicals, might be involved in alcohol breast cancer induction. This might be the case, particularly also in cases of a high consumption of purine-rich food (e.g. meat) or beverages or soft drinks containing caffeine.
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1. Introduction

Cancer of the breast is the third most common cancer in the world and the most common cancer in women. Over 50% of breast cancer incidence occurs in the developed world, and populations

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that migrate from low- to high-incidence countries develop rates of breast cancer that approximate to those of the new host country, although the rapidity with which this occurs varies (World Cancer Research Fund/American Institute for Cancer Research, 1997; World Health Organization, 1997; Parkin, 1998).

This suggests that environmental factors would be related to breast cancer. Diet appears to be a relevant factor. For example, diets high in total fat possibly increase the risk of cancer (National Academy of Sciences, 1982, 1989; World Health Organization, 1990; World Cancer Research Fund/American Institute for Cancer Research, 1997). Evidence suggesting that diets high in animal protein may increase the risk of breast cancer is available but so far has been considered insufficient (World Cancer Research Fund/American Institute for Cancer Research, 1997). Estrogenic food additives, consumption of alcoholic beverages and the presence of estrogenic food contaminants have been linked to a risk of breast cancer (Nicholson, 1996; Wolf and Weston, 1997; World Cancer Research Fund/American Institute for Cancer Research, 1997). Other factors associated with risk of breast cancer are also indicative of endogenous hormonal contribution (White, 1987; Kampert et al., 1988; Kelsey et al., 1993; Reichmen et al., 1993; Lilienfield, 1996; Singletary, 1997). Some of the latter observations have implicated endogenous hormone, particularly estrogens as underlying biological determinants of breast cancer incidence (Pike et al., 1983). Notwithstanding, it is thought that hormones fundamentally play a promotional role in breast carcinogenesis stimulating mitotic division of already initiated cells (World Cancer Research Fund/American Institute for Cancer Research, 1997). However, the nature of the mutational event responsible for the initiation step of the process is not clear. A role for chemical carcinogens in breast cancer is supported by the analysis of the mutational spectrum of the p53 gene since the pattern of its mutation in breast cancer is quite similar to that for lung cancer in which chemical carcinogens are known to be etiologically related (Biggs et al., 1993). Numerous animal studies have identified chemical carcinogens that result in mammary tumors in rodents, but no known human carcinogens have been clearly established (Wolf and Weston, 1997; World Cancer Research Fund/American Institute for Cancer Research, 1997). The best-defined risk factors for breast cancer in humans are radiation exposure and alcohol ingestion (Wolf and Weston, 1997; World Cancer Research Fund/American Institute for Cancer Research, 1997). There is at present considerable evidence from epidemiological studies to support a positive association between alcohol intake and risk for breast cancer. However, there is limited information regarding possible mechanisms for this effect (Mufti, 1992; Hankinson et al., 1995; Holmberg et al., 1995; Longnecker et al., 1995a,b; Van den Brandt et al., 1995; Halke, 1996; Hunter and Willett, 1996; Levi et al., 1996; Nicholson, 1996; Segvardsson et al., 1996; Kohlmeier and Mendez, 1997; Wolf and Weston, 1997; World Cancer Research Fund/American Institute for Cancer Research, 1997). In the present studies, we describe initial experiments to test the possibility that breast cytosolic xanthine oxidoreductase (xanthine oxidase + xanthine dehydrogenase) (XOR = XDh + XO) is able to bioactivate ethanol to acetaldehyde and/or free radicals.

2. Materials and methods

2.1. 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 [NAD^+; hypoxanthine, xanthine, caffeine, theobromine, theophylline, 1,7-dimethylxanthine, allopurinol, desferrioxamine mesylate (DFA), superoxide dismutase from bovine erythrocytes (SOD), etc.] were from Sigma Co.

2.2. Animals and treatments

Non-inbred female Sprague–Dawley rats (220–260 g) were used: these were post-lactation young mothers (2 weeks after weaning). The animals were starved for 12–14 h before sacrifice. Water

was available ad libitum. Animals were killed by decapitation, and their breasts were rapidly excised and processed. Purified cytosolic fractions were obtained as previously described and were essentially free from cross-contamination (Masana et al., 1984). Phenylmethylsulfonyl fluoride (PMSF, 1 mM) was added to the homogenizing buffer in order to avoid the irreversible conversion (by proteolysis) of XDh to XO. We did not use dithioerythritol (DTE) in the cytosol preparation procedure because in that case, conversion to the XDh form is almost quantitative (Terada et al., 1990).

2.3. Ethanol biotransformation to acetaldehyde in the cytosolic fraction

Incubation mixtures containing purified cytosol (3.80 ± 0.29 mg protein per ml) 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 conducted for 1 h at 37°C under an air atmosphere. In order to test the presence of the XO enzymatic system, hypoxanthine (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). The reaction was interrupted by cooling.

After adding 1 ml of saturated NaCl solution, samples were thermostatized at 40°C for 10 min and an aliquot (100 μl) of the headspace analyzed by GC-FID.

Chromatographic conditions were: column, Poraplot Q, 25 m × 0.53 mm i.d. (Chrompack, The Netherlands); temperature: 140°C isothermal, injection port temperature: 150°C, FID: 200°C (Castro et al., 1997, 1998 Díaz Gómez et al., 1999).

2.4. Free-radical determination in "in-vitro" biological systems

The formation of free radicals was investigated by the method described previously (Castro et al., 1997, 1998 Castro and Castro, 2000). Briefly, in experiments involving cytosolic activation of ethanol, selected ion monitoring (SIM) of mass spectrum of adducts was employed to increase sensitivity. Selected masses were 250 and 194 (Castro and Castro, 2000). In experimental condition I, purified cytosolic fractions (4.5 ± 0.3 mg cytosolic protein per ml) were added to 25 mM PBN, 0.25 mM hypoxanthine and, when indicated, 0.21 M ethanol. In the experimental condition II, incubations contained the same plus 50 μM $FeCl_3$ and 24 U/ml of SOD. Incubation condition III corresponded to the additional presence of NAD^+ (0.3 mM) After 1 h at 37°C, the volume (3 ml) was extracted with 500 μl of toluene, centrifuged, and the organic layer evaporated under nitrogen. The residue was silylated with BSTFA:acetonitrile (1:1), 60°C for 15 min and analyzed by GC/MS-SIM. The 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. The injection port was at 250°C and the transfer line to MS at 300°C. The dwell time was 50 ms for both masses selected.

3. Results

3.1. Ethanol biotransformation to acetaldehyde in cytosolic fraction

Results on acetaldehyde levels in incubation mixtures containing the cytosolic fraction of rat breast tissue are summarized in Table 1. The reaction was sensitive to heating (5 min at 100°C), enhanced by the presence of NAD^+ but not inhibited by pyrazole. Instead, allopurinol was able to inhibit the acetaldehyde formation.

The ethanol metabolism to acetaldehyde was significantly enhanced by 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. In all cases, there was an additional enhancing effect of NAD^+ on acetaldehyde levels. Those processes were also inhibited by allopurinol. Replacement of purines by *N*-methylnicotinamide did not significantly en-

hance the ability of breast cytosol to biotransform ethanol to acetaldehyde.

Table 1
Ethanol biotransformation to acetaldehyde by breast cytosol

Experimental[a]	Acetaldehyde (ng)/protein (mg)
Control	4.55 ± 0.20
0.3 mM NAD^+	73.79 ± 1.82
Heated (100°C, 5 min)	0.89 ± 0.08
NAD^+ + 5 mM pyrazol	71.72 ± 0.83
NAD^+ + 0.15 mM allopurinol	3.29 ± 0.38
Allopurinol	4.39 ± 0.22
Hypoxanthine	116.50 ± 0.79
Hypoxanthine + allopurinol	4.77 ± 0.53
Hypoxanthine + NAD^+	216.67 ± 19.48
Hypoxanthine + NAD^+ + allopurinol	4.44 ± 0.98
Heated + hypoxanthine + NAD^+	3.29 ± 0.64
Heated + hypoxanthine + NAD^+ + allopurinol	4.11 ± 0.28
Xanthine + NAD^+	190.81 ± 5.94
Xanthine + NAD^+ + allopurinol	2.71 ± 0.15
Caffeine + NAD^+	135.13 ± 2.28
Caffeine + NAD^+ + allopurinol	4.59 ± 0.60
Theobromine + NAD^+	136.60 ± 5.04
Theobromine + NAD^+ + allopurinol	3.32 ± 0.68
Theophylline + NAD^+	144.97 ± 6.27
Theophylline + NAD^+ + allopurinol	2.57 ± 0.60
1,7-dimethylxanthine + NAD^+	144.01 ± 2.81
1,7-dimethylxanthine + NAD^+ + allopurinol	4.33 ± 0.45
Hypoxanthine + NAD^+ + 25 mM Na benzoate	241.74 ± 1.12
Hypoxanthine + NAD^+ + 25 mM Na benzoate + allopurinol	4.81 ± 0.49
Hypoxanthine + NAD^+ + 1 mM DFA	211.85 ± 7.87
Hypoxanthine + NAD^+ + 1 mM DFA + allopurinol	6.32 ± 0.29
2.5 mM *N*-methylnicotinamide	3.25 ± 0.04
N-methylnicotinamide + 10 μM menadione	4.55 ± 0.48

[a] Incubation mixtures containing cytosol (3.80 ± 0.29 mg of cytosolic protein/ml), 0.21 M ethanol and, when indicated, 0.25 mM hypoxanthine (xanthine, caffeine, theobromine, theophylline or 1,7-dimethylxanthine), 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 Section 2 for details. Each result is the mean of three separate samples.

3.2. Free-radical detection in in-vitro experiments with a breast cytosolic fraction

Fig. 1 shows the capillary GC analysis with SIM detection of reaction products arising from ethanol biotransformation by a breast cytosolic fraction in the presence of the spin-trap PBN. No peak arising from 1HEt radical was observed, even when iron, SOD or NAD^+ was present in incubations. Instead, two peaks due to hydroxylation of PBN were observed (peaks A and B in Fig. 1a). These compounds were reported previously as being formed in the prostate cytosolic or liver microsomal biotransformation of ethanol (Castro et al., 1998, 2000 Castro and Castro, 2000). Hydroxyl addition to the aromatic ring of the spin trap can account for the proposed structures in the case of compounds A and B (Castro and Castro, 2000; Reinke et al., 2000). In the presence of $FeCl_3$/SOD (incubation condition II), there was a slight increase of peaks A and B, but it was NAD^+ (incubation condition III) that induced a significative enhancing effect on the formation of hydroxylated PBN peaks (Fig. 1b). This effect was reverted to background levels by allopurinol (Fig. 1c). The presence of ethanol in incubation media decreased the formation of peaks A and b, but this was not accompanied by the formation of the 1HEt adduct of PBN (Fig. 1d). Allopurinol was also able to inhibit this process (Fig. 1e).

4. Discussion

The obtained results evidence that the cytosolic fraction of rat breast tissue has the ability to biotransform ethanol to acetaldehyde. In effect, as shown in Table 1, breast cytosol in the presence of NAD^+ produces acetaldehyde from ethanol. The process involved is enzymatic in nature since heating for 5 min at 100°C fully supresses that activity. The biotransformation process is not inhibited by pyrazole, and that excludes the participation of a cytosolic alcohol dehydrogenase, since pyrazole is a well-known inhibitor of this enzyme (Li and Theorell, 1969). The ability of cytosol to use NAD^+ and its susceptibility to allopurinol inhibitory effects would indicate that XDh is partic-

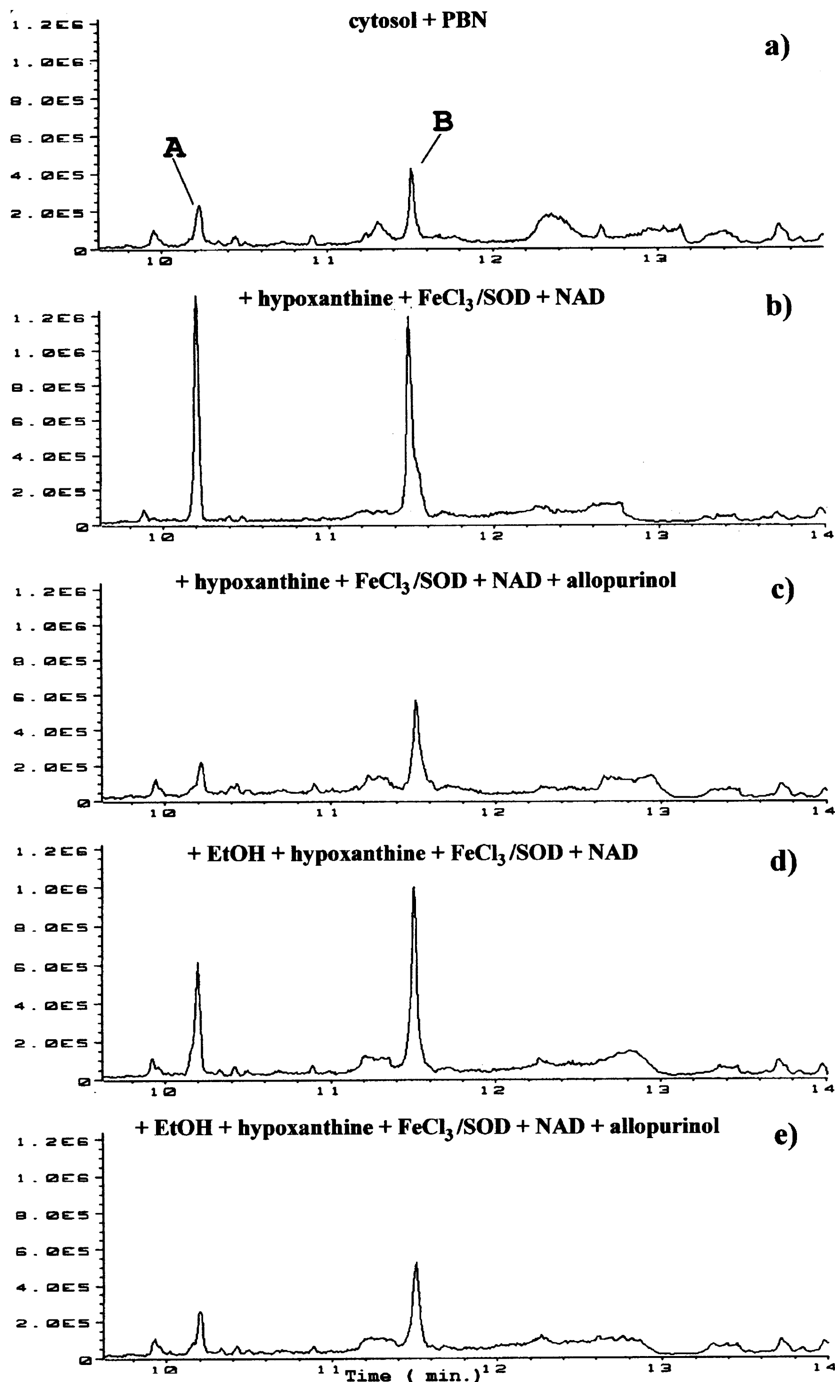

Fig. 1. (a) Selected-ion current profile obtained from GC–MS–SIM analysis of a sample from incubation containing rat breast cytosol, in the presence of 25 mM *N*-*t*-butyl-α-phenylnitrone (PBN), after trimethylsilylation. Peaks: A and B, aromatic hydroxylated PBN derivatives. See Section 2 for other details. (b) With 0.25 mM hypoxanthine, 50 μM $FeCl_3$, 24 U/ml of SOD and 0.3 mM NAD^+. (c) Same as in (b) in the presence of 0.15 mM allopurinol. (d) Same as in (b) plus 0.21 M ethanol. (e) Same as in (d), in the presence of 0.15 mM allopurinol.

ipating in the ethanol biotransformation. In effect, allopurinol is a well-known inhibitor of both XDh and XO (Rajagopalan, 1980; Masana et al., 1984; Kato et al., 1990). The additional participation of the XO form of the enzyme in the ethanol oxidation to acetaldehyde by breast cytosol is further evidenced by the ability of the process to occur when NAD^+ is accompanied by substrates/cosubstrates of the XO form such as hypoxanthine, xanthine, caffeine, theobromine, theophylline, and 1,7-dimethylxanthine (Kato et al., 1990). That is, both XDh and XO are able to participate in the ethanol biotransformation to acetaldehyde when the reaction is performed in the presence of substrates of both NAD^+ and hypoxanthine, for example, when a potentiation effect is observed. The susceptibility of the occurring reactions to allopurinol, but not to a hydroxyl radical trapping agent such as benzoate, further confirms this hypothesis (Halliwell and Gutteridge, 1991). The presence of both enzymes in breast tissue is very well known (Dieter-Jarasch et al., 1981; Kooij et al., 1991). The potential participation of any iron in the cytosol does not appear to play any role because acetaldehyde formation from ethanol was not decreased by the presence of DFA in the incubation mixture. DFA is a potent iron chelator and inhibitor of many iron-mediated oxidative processes (Halliwell, 1985). When substrates for XOR activity are replaced in the incubation by a selective substrate of another molybdoenzyme aldehydeoxidase (AO) such as is *N*-methylnicotinamide, the oxidation of ethanol does not proceed significantly, and the small acetaldehyde formation occurring under those conditions is not inhibited by menadione, a potent inhibitor of AO (Rajagopalan, 1980; Masana et al., 1984). Under standard incubation conditions (incubation condition a), we failed to detect the formation of either hydroxyl or 1HEt free radicals during cytosolic XOR biotransformation of ethanol. However, we detected aromatic hydroxylated PBN derivatives. These compounds were previously reported to be formed under experimental conditions where hydroxyl radicals are being produced (Castro et al., 1998, 2000 Castro and Castro, 2000 Reinke et al., 2000). Notwithstanding, they might also arise during microsomal metabolism of PBN (Castro and Castro, 2000 Reinke et al., 2000). The feasibility of an equivalent behavior of XOR acting on PBN to yield similar products is not known. Equivalent studies using XOR-containing cytosolic fractions from prostate, however, led to hydroxyl and 1HEt radical formation (Castro et al., 2000). The presence of effective free-radical scavengers in breast cytosol might be one potential explanation for these apparently contrasting observations. Lack of sensitivity of our detection system might be another explanation. A combination of both factors is also possible. We obtained some indication that this might be the case in our experiments, where optimized incubation mixtures (incubation conditions b and c), rather than the standard incubation mixture, were used. In such incubation mixtures, increased XOR activity was reached by using NAD^+ plus hypoxanthine, and the trapping efficiency was increased by employing a high PBN concentration. Under these circumstances, metabolism of ethanol by XOR was accompanied of detectable formation of aromatic hydroxylated PBN derivatives. Concerning the toxicological significance of these findings is the fact that breast cytosol XOR is able to bioactivate ethanol to acetaldehyde and potentially to hydroxyl radicals formation. The former is a mutagenic and carcinogenic chemical (Woritersen et al., 1984, 1985; International Agency for Research on Cancer, 1985; Dellarco, 1988), and hydroxyl radicals are considered to be involved in the initiation and promotion stages of ethanol-induced cancers in different tissues. Further, acetaldehyde is also known to be able to inhibit O^6-methyl guanine demethylase, which is known to be critical for the repair of O^6-methyl guanine lesions in DNA caused by *N*-nitrosodimethylamine and of other equivalent alterations resulting from other alkylating carcinogens (Garro et al., 1986; Mufti et al., 1988). It is interesting that acetaldehyde and potentially hydroxyl-radical formation can be significantly enhanced by purines, which are cosubstrates of XOR (Rajagopalan, 1980; Masana et al., 1984). In effect, diets rich in purines (e.g. meat products) might enhance cosubstrate supply in the form of purine degradation products, e.g. xanthine/hypoxanthine (Rajagopalan, 1980). Fur-

ther, different beverages such as coffee and some soft drinks may also be sources of caffeine and its degradation products (Rajagopalan, 1980). Moreover, it is also known that during acute alcohol intoxication, there is an increased purine degradation and hyperuricemia with increased levels in blood and liver of NADH/xanthine and hypoxanthine (Kato et al., 1990). That is, the simultaneous presence of both a cosubstrate and alcohol may enhance significantly the formation in breast tissue of the mutagen/carcinogen acetaldehyde. The other molybdenum-containing metalloenzyme able to use some other purine substrates, aldehyde oxidase (Rajagopalan, 1980), was not found in breast tissue. Whether the biotransformation of ethanol to reactive intermediates reported here is an additional contributory factor to promotion of breast cancer, in conjunction with others of hormonal or other nature, however, remains to be established and should be considered only a working hypothesis.

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