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Ethanol-induced oxidative stress and acetaldehyde formation in rat mammary tissue: Potential factors involved in alcohol drinking promotion of breast cancer☆

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Abstract

Recent studies from our laboratory provided evidence that part of the carcinogenic effects of ethanol consumption might be related to its in situ metabolism at cytosolic and microsomal levels, to the mutagen acetaldehyde and to hydroxyl and 1-hydroxyethyl radicals. In this work, we report on our experiments where Sprague–Dawley female rats were exposed to the standard Lieber & De Carli diet for 28 days. We observed: the induction of the (xanthineoxidoreductase mediated) cytosolic and microsomal (lipoxygenase mediated) pathways of ethanol metabolism; promotion of oxidative stress as shown by increased formation of lipid hydroperoxides; delay in the *t*-butylhydroperoxide induced chemiluminiscence, and a significant decrease in protein sulfhydryls. In addition, the epithelial cells showed ultrastructural alterations consisting of markedly irregular nuclei, with frequent invaginations at the level of the nuclear envelope, condensation of chromatin around the inner nuclear membrane, and marked dilatation of the nuclear pores showing filamentous material exiting to the cytoplasm. In conclusion, the presence in mammary epithelial cells of cytosolic and microsomal pathways of ethanol bioactivation to carcinogenic and to tumorigenic metabolites might play a role in alcohol promotion of breast cancer.

Keywords: Breast; Mammary, Alcohol; Ethanol; Lipoxygenase; Xanthine oxidoreductase; Cancer

1. Introduction

There is abundant evidence in literature about chronic alcohol (EtOH) consumption associated with a modest increased risk of breast cancer, even if consumed in moderate doses. According to estimates of the World Health Organization, about 3% of breast cancer worldwide were attributable to EtOH consumption in 1990. Combined analysis of data from 53 studies around the world showed a clear dose–response relationship between EtOH consumed and increased in risk of breast cancer, leading

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to the estimation that risk increases 7% for each additional 10 g of EtOH consumed daily (reviewed in Stewart and Kleihues, 2003; Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

However, the biological and molecular mechanisms mediating this association remain unknown and speculative. Several studies support the hypothesis that EtOH use may increase breast cancer risk at least partially through an effect in sex steroid levels. In fact, EtOH drinking results in higher estrogen levels in women (reviewed by Ginsburg, 1999).

There is considerable evidence that risk of breast cancer is related to cumulative estrogen exposure and that prolonged stimulation by the steroid hormone increases cell division. Increased cell proliferation per se, stimulated by external or internal factors, was hypothesized by some workers to be sufficient to develop many human cancers (reviewed by Preston-Martin et al., 1990). However, most results available in literature led to workers in the field to consider that hormone mediated mitogenic effects of EtOH on breast epithelial cells play a promotional role in breast carcinogenesis essentially by stimulating mitotic division of already initiated cells (Przylipiak et al., 1996; Singletary et al., 2001; Izevbigie et al., 2002; Izevbigie, 2005; Etique et al., 2004; Coutelle et al., 2004; Dumitrescu and Shields, 2005). Under this assumption, the nature of the mutational events responsible for the initiation step of the process would remain unclear. In addition, recent results from our and other laboratories showed that effects of EtOH in the process leading to breast cancer may not necessarily be only indirect, via increased levels of estrogen. For example, our laboratory recently demonstrated that rat mammary gland cytosolic xanthine oxidoreductase (XOR) is able to bioactivate EtOH to acetaldehyde (AC) and free radicals (Castro et al., 2001) and that the microsomal fraction exhibited the presence of a lipoxygenase mediated enzymatic pathway for the metabolism of EtOH to AC (Castro et al., 2003). This might be of relevance considering that no alcohol dehydrogenase activity was found in homogenates of rat mammary tissue (Guerri and Sanchis, 1986). In addition, Triano et al. (2003) reported that the cytosolic fraction from human mammary tissue contains a class I alcohol dehydrogenase oxidizing EtOH at concentrations ranging from 0.05 to 4.0 mM but inhibiting it at higher concentrations of EtOH. Using highly specific and sensitive reverse transcriptase-polymerase chain reaction analysis or immunohistochemistry there were reports about CYP2E1 presence in human breast tumor and non-tumor tissues (Iscan et al., 2001; Kapucuoglu et al., 2003). In liver, the significant presence of microsomal CYP2E1 is known to be relevant in the production of acetaldehyde, 1-hydroxyethyl radicals and reactive oxygen species which in turn, via lipid peroxidation, can promote the formation of 4-hydroxynonenal. This compound reacts with DNA to lead to exocyclic adducts (Frank et al., 2004). It has not been established yet whether a similar CYP2E1 mediated process might occur in breast tissue exposed to alcohol. Notwithstanding, any contribution of cytochrome P450 to rat breast microsomal fraction metabolism of ethanol was found to be in our hands quantitatively not significant (Castro et al., 2003). In summary, these results showed that the mammary tissue may bioactivate EtOH in situ to the highly mutagenic AC (IARC, 1985; Dellarco, 1988) and that it is able to generate free radicals, a process potentially leading to oxidative stress, known to play a role in tumor promotion (Hussain et al., 2003; Castro and Castro, 2004). Generation of AC and oxidative stress sparked during EtOH bioactivation were considered key factors in the genesis of the hepatocarcinogenic effects of EtOH (Garro and Lieber, 1990; Pöschl et al., 2004) and that suggests that they might also play a significant role in the EtOH promotion of breast cancers in addition to the relevant effects of estrogen on cell proliferation.

In the present work, we show that repetitive alcohol drinking enhances the activity of the pathways of metabolic activation of EtOH to AC and free radicals at cytosolic and microsomal level and promote oxidative stress in mammary tissue.

2. Materials and methods

2.1. Chemicals

EtOH (analytical grade) and methanol (HPLC-grade) was from Sintorgan (Argentina). Hypoxanthine, allopurinol, NAD^+, $NADP^+$, 2,4-dinitrophenylhydrazine and deferoxamine mesylate were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the best quality available.

2.2. Animals

Non-inbred female 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).

In the treatment with EtOH liquid diet, Sprague–Dawley female rats (125–150 g body weight, 5–6 weeks age) were fed with a nutritionally adequate liquid diet (Lieber & De Carli standard rat diet, purchased from Dyets Inc., PA). The rats were housed in individual cages and separated into two dietary groups: EtOH group (EtOH) and Control group (Con-

trol). Both groups were pair fed with the same diet except that in Control, 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 with the Control and EtOH diets was continued for 28 days. The amount of EtOH was started with 30 g/l of the liquid diet for the first 2 days, 40 g/l for the subsequent 2 days followed by the final formula containing 50 g/l.

2.3. Isolation of cytosolic and microsomal fractions

Animals were killed by decapitation and their mammary tissue was rapidly excised and processed to obtain cytosolic and microsomal fractions. Cytosolic and microsomal fractions were obtained from whole mammary tissue homogenates by cellular fractionation procedures via ultracentrifugation at 4 °C (Castro et al., 2003; Masana et al., 1984).

2.4. Ethanol metabolism to acetaldehyde in the cytosolic fraction

Incubation mixtures containing cytosol (1.58–1.65 mg protein/ml) in STKM buffer (0.25 M sucrose/50 mM Tris–HCl, pH 7.5/2.5 mM KCl/5 mM $MgCl_2$); 0.25 mM hypoxanthine; 0.3 mM NAD^+ and 0.14 M EtOH (3 ml final volume), were conducted for 1 h at 37 °C under air atmosphere. Three samples per group were run, each consisting of cytosol prepared from a separate lot of pooled mammary tissue (three animals each). Incubations were performed in aluminum-sealed neoprene-septum stoppered glass vials (15 ml). The reaction was terminated by plunging in ice. After adding 1 ml of saturated NaCl solution, samples were kept at 37 °C for 10 min and an aliquot (100 μl) of the headspace was analyzed by GC-FID. Chromatographic conditions were: column, GS-Q, 25 m × 0.53 mm i.d. (J&W Scientific, CA); temperature 110 °C isothermal, injection port temperature: 150 °C, FID: 200 °C (Castro et al., 1998, 2003; Díaz Gomez et al., 1999).

2.5. Ethanol metabolism to acetaldehyde in the microsomal fraction

Preparations containing microsomes (0.23–0.32 mg 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 0.14 M EtOH in 50 mM KH_2PO_4, pH 7.4, 3 ml final volume, were incubated for 1 h at 37 °C under air. Three samples per group were run, each consisting of microsomes from a separate lot of pooled mammary tissue (three animals each). Incubations were performed in aluminium-sealed neoprene-septum-stoppered glass vials. Samples were processed as described above. AC was analyzed in the head space by GC-FID in the same conditions as in Section 2.4.

2.6. Histochemical procedure for xanthine oxidase activity detection in mammary tissue

Portions of mammary tissue from Control rats (five animals) were frozen at −70 °C in hexane in a mixture of solid carbon dioxide and absolute alcohol. Tissue blocks were stored at −80 °C until further use. Sections 8 μm thick were cut on an International Cryostat IEC at −24 °C. The sections were picked up onto clean glass slides and incubated immediately for xanthine oxidase activity using the cerium capture method in the presence of polyvinyl alcohol described by Frederiks et al. (1994). Briefly, the incubation mixture contained 100 mM Tris–maleate buffer, pH 8; 10 mM cerium chloride; 100 mM sodium azide; 0.5 mM hypoxanthine and 10% polyvinyl alcohol. Incubations lasted 60 min at 37 °C. After that, sections were washed in hot distilled water (60 °C). Visualization was performed by incubating sections for 30 min at room temperature in 50 mM acetate buffer, pH 5.3; 42 mM cobalt chloride; 100 mM sodium azide; 1.4 mM diaminobenzidine and 0.6 mM H_2O_2. After rinsing the sections were embedded in glycerol jelly.

2.7. Determination of t-butylhydroperoxide induced chemiluminiscence in rat mammary tissue homogenates

Chemiluminiscence was measured in a Wallac-Rack Beta 1214 liquid scintillation counter at room temperature in an out of coincidence mode (Boveris et al., 1983). Rat mammary tissue was homogenized (7 mg protein/ml) in 0.25 M sucrose, 50 μM deferoxamine in TKM buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM KCl), pH 7.5 in flasks that were kept at 37 °C for 10 min in a Dubnoff shaker. Chemiluminiscence measurement was started by addition of 3 mM *t*-butylhydroperoxide. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each).

2.8. Measurement of lipid hydroperoxides by the xylenol orange method in mammary tissue from rats receiving an EtOH containing liquid diet

Rat mammary tissue was homogenized in cold HPLC-grade methanol, the 1000 × *g* supernatants were used for lipid hydroperoxide determination, using the xylenol orange method (Eymard and Genot, 2003). In order to discern color development due to authentic lipid hydroperoxide from that due to H_2O_2 or other interfering components, 0.1 mM triphenylphosphine (TPP), a specific hydroperoxide reductant that has no effect on H_2O_2, was added to a set of vials to reduce lipid hydroperoxides (Nourooz-Zadeh et al., 1994). The samples were incubated with the ferrous oxidation-xylenol orange reagent (100 μM xylenol orange; 0.25 mM ammonium ferrous sulphate hexahydrate; 25 mM H_2SO_4) in darkness at room temperature, until the reaction was complete (270 min). Four samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). Absorbance of the xylenol complex was measured at 560 nm

every 30 min. Levels of lipid hydroperoxides were determined as the difference in vials with and without TPP. A standard curve was developed with *t*-butylhydroperoxide (TBHP) and the levels of lipid hydroperoxides were expressed as nmol of TBHP equivalents/g of mammary tissue.

2.9. Protein carbonyl content determination

Rat mammary tissue was homogenized in 0.15 M Tris–HCl, pH 7.4, 1 mM KH_2PO_4. Protein carbonyl was carried out in $600 \times g$ supernatants by the 2,4-dinitrophenylhydrazine technique (Levine et al., 1990). Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). Carbonyl content was calculated from the spectrophotometric absorbance at 370 nm, using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} (Galelli et al., 1997).

2.10. Protein sulfhydryl content determination

Rat mammary tissue was homogenized as described in carbonyl determination. Protein sulfhydryl determination was carried out in $600 \times g$ supernatants using Ellman's reagent according to the Jocelyn technique (Levine et al., 1990). Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). Sulfhydryl content was calculated from the absorbance at 412 nm, using a molar absorption coefficient of 13,100 M^{-1} cm^{-1} (Galelli and Castro, 1998).

2.11. Determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)

Approximately 500 mg of mammary tissue was used to extract DNA using the chaotropic method and the DNA obtained was hydrolyzed with Nuclease P1 and alkaline phosphatase, essentially as described by Ravanat et al. (2002). Five animals per group were analyzed. The measurement of 8-oxodGuo was carried out in a Hewlett-Packard 1090 HPLC coupled to a Coulochem III electrochemical detector (ESA, Chelmsford, MA). The isocratic mobile phase was 50 mM KH_2PO_4 (pH 4.5), 8% methanol. Separation of the nucleosides was performed using a C_{18} reversed-phase column (Spherisorb ODS-2 5 μm, 250 mm × 4.6 mm, Sigma–Aldrich). The 8-oxodGuo levels were expressed as the ratio of 8-oxodGuo per 10^5 dGuo (Pouget et al., 2000). All samples were analyzed in triplicate.

2.12. Protein concentration determination

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

2.13. Transmission electron microscopy

Five female rats per group (Control and EtOH treated animals) were anesthetized by diethyl ether. The mammary gland was rapidly removed and immediately placed in chilled 2% formaldehyde: 2% glutaraldehyde in 100 mM cacodylate buffer containing 0.02% $CaCl_2$, pH 7.4, and promptly cut under the fixative. After adequate fixation, 10 cubes (1 mm^3) per each rat mammary gland were washed with barbital buffer and post fixed with 1% osmiun tetroxide. Then, they were stained as a whole with uranyl acetate, dehydrated with graded EtOH and embedded in epoxy resin. Sections 1 μm thick were stained with toluidine blue and examined with a light microscope in order to select epithelial areas for thin sectioning. Thin sections were cut with a diamond knife and mounted on copper grids (300 mesh), stained with uranyl acetate and lead citrate and examined in a Philips EM300 transmission electron microscope (de Castro et al., 2003).

2.14. Statistics

The significance of the difference between 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 $P < 0.05$.

3. Results

3.1. Ethanol metabolism to acetaldehyde in the mammary tissue cytosolic fraction from rats receiving an alcohol containing liquid diet

In cytosolic fractions from rats repetitively receiving an alcohol containing liquid diet, the NAD^+ and hypoxanthine dependent metabolism of EtOH to AC was significantly more intense than in pair fed Controls (Table 1). The metabolic activity in both experimental groups was strongly inhibited by allopurinol, the inhibitor of XOR.

3.2. Ethanol metabolism to acetaldehyde in the mammary microsomal fraction from rats receiving an alcohol containing liquid diet

Both, a microsomal NADPH dependent and a NADPH independent microsomal metabolism of EtOH to AC were observed (Table 2). The former was significantly more intense than the latter. After chronic EtOH drinking the activity of the NADPH dependent pathway was significantly enhanced (27.1%). The metabolic pathway independent of NADPH presence was also enhanced significantly (25.8%).

3.3. Xanthine oxidase activity in rat mammary tissue

Histochemical studies showed that xanthine oxidase activity was present in high amounts in control mammary tissue epithelial cells (Fig. 1).

Table 1
Ethanol metabolism to acetaldehyde in the cytosolic fraction of mammary tissue from rats receiving an alcohol containing liquid diet

Experimental[a]	Acetaldehyde (nmol/mg protein)	
	Control	EtOH treated
Hypoxanthine + NAD	0.77 ± 0.01	1.38 ± 0.04[b]
Hypoxanthine + NAD + allopurinol	0.02 ± 0.01[b]	0.05 ± 0.01[b,c]

[a] Incubation mixtures containing cytosolic fraction (1.58–1.65 mg protein/ml), 0.14 M ethanol and, when indicated, 0.25 mM hypoxanthine and 0.3 mM NAD^+ in STKM buffer, 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 lots of pooled mammary tissue samples.
[b] $P < 0.0001$ (EtOH treated + NAD + hypoxanthine vs. Control + NAD + hypoxanthine) (+NAD + hypoxanthine vs. +NAD + hypoxanthine + allopurinol).
[c] $P < 0.005$ (EtOH treated + NAD + hypoxanthine + allopurinol vs. Control + NAD + hypoxanthine + allopurinol).

Table 2
Ethanol metabolism to acetaldehyde in the microsomal fraction of mammary tissue from rats receiving an alcohol containing liquid diet

Experimental[a]	Acetaldehyde (nmol/mg protein)	
	Control	EtOH treated
+NADPH	1.51 ± 0.03	1.92 ± 0.01[b]
−NADPH	0.56 ± 0.02[c]	0.71 ± 0.03[b]

[a] Incubation mixtures containing microsomal fraction (0.23–0.32 mg protein/ml), 0.14 M ethanol and, when indicated, NADPH generating system (0.45 mM $NADP^+$, 4 mM D,L-isocitric acid trisodium salt and 0.25 units of isocitric dehydrogenase) in KH_2PO_4 buffer, 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 lots of pooled mammary tissue samples.
[b] $P < 0.0001$ (EtOH treated vs. Control) (EtOH treated − NADPH vs. EtOH treated + NADPH).
[c] $P < 0.0005$ (Control − NADPH vs. Control + NADPH).

3.4. *t*-Butylhydroperoxide induced chemiluminiscence in homogenates of mammary tissue from rats receiving an alcohol containing liquid diet

In our studies on the *t*-butylhydroperoxide induced chemiluminiscence in rat mammary tissue homogenates, we observed that homogenates from Control rats showed a delayed response in chemiluminiscence to the challenging hydroperoxide when compared to those receiving the EtOH containing diet (Fig. 2).

3.5. Lipid hydroperoxides in mammary tissue from rats receiving an alcohol containing liquid diet

The levels of lipid hydroperoxides, expressed as TBHP equivalents, were measured in Control as well

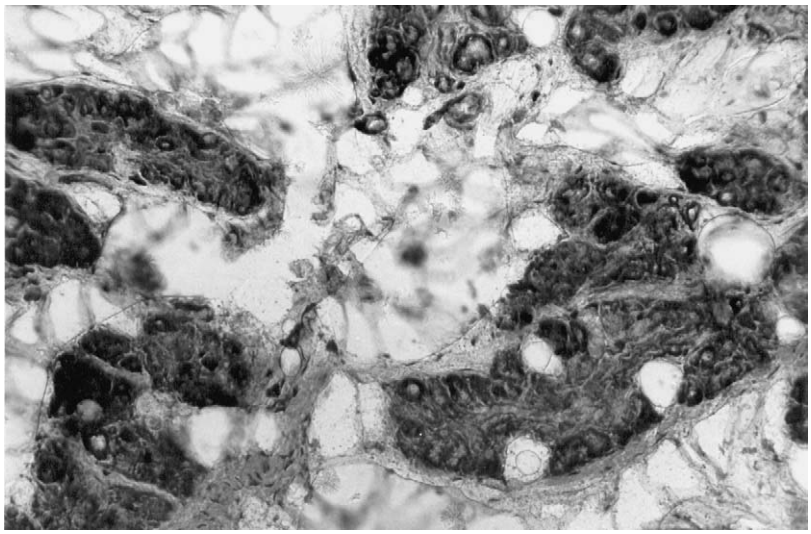

Fig. 1. Xanthine oxidase activity in epithelial cells of rat mammary gland. Section of rat mammary tissue showing positive staining for the presence of xanthine oxidase activity. 160×. See Section 2 for details.

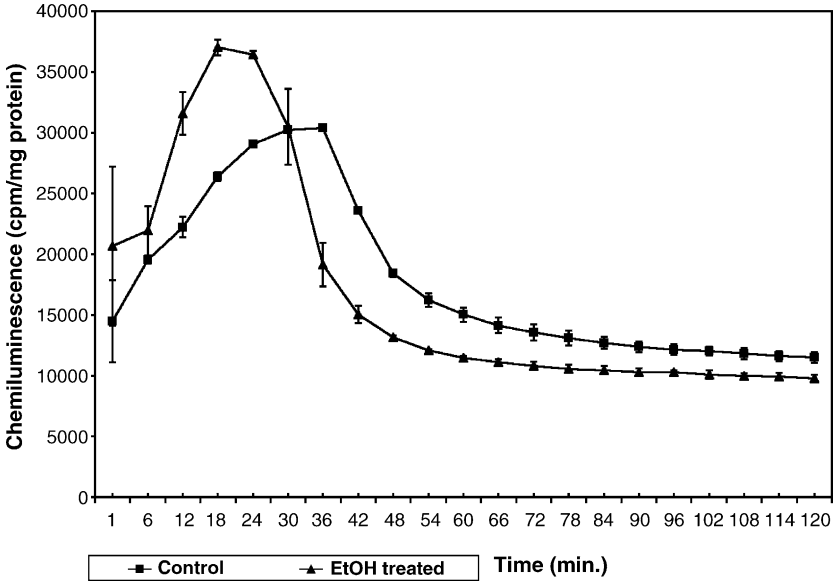

Fig. 2. *t*-Butylhydroperoxide induced chemiluminiscence in rat mammary tissue homogenates from rats receiving an alcohol containing liquid diet. Rat mammary tissue homogenized in 0.25 M sucrose, 50 μM deferoxamine in TKM buffer, pH 7.5 (7 mg protein/ml) were kept at 37 °C for 10 min in a Dubnoff shaker. Chemiluminiscence measurement was started by addition of 3 mM *t*-butylhydroperoxide. Values are the means ± S.D. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each).

as in rats treated with the alcohol liquid diet. After incubating with the reagent and subtracting the possible formation of hydroperoxides due to H_2O_2, a significant higher level of lipid hydroperoxides in the EtOH treated rats as compared to Controls was observed (see Fig. 3).

3.6. Protein sulfhydryl, protein carbonyl and 8-oxodGuo content in DNA of mammary tissue from rats receiving an alcohol containing liquid diet

Mammary tissue from rats receiving the EtOH containing liquid diet exhibited a significant decrease in its

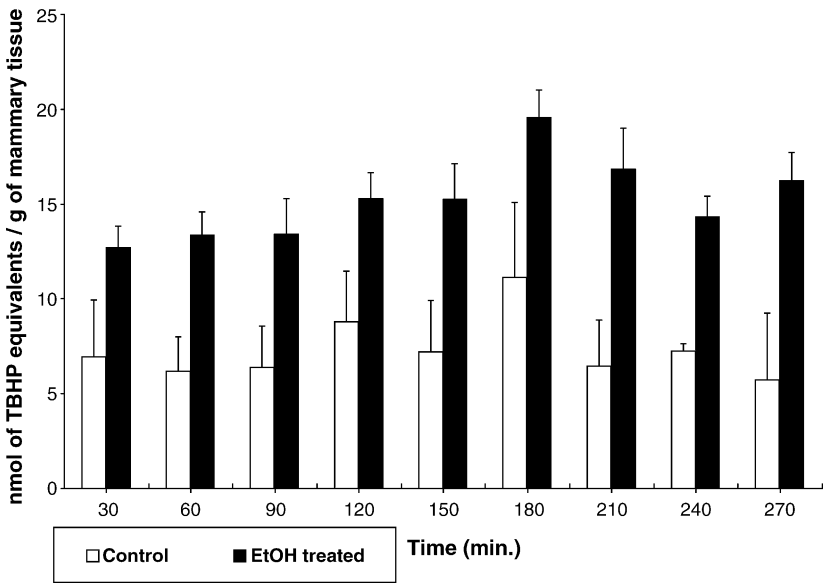

Fig. 3. Lipid hydroperoxides in mammary tissue from rats receiving an alcohol containing liquid diet. Rat mammary tissue extracts were incubated with the xylenol orange reagent at room temperature for different times, as described in Section 2. Values are the means ± S.D. Four samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each).

Table 3
Protein sulfhydryl, protein carbonyl and 8-oxodGuo content in DNA of mammary tissue from rats receiving an alcohol containing liquid diet

	nmol C=O/mg protein[a]	nmol SH/mg protein[b]	8-oxodGuo/10^5 dGuo[c]
Control	5.44 ± 0.45	90.48 ± 4.27	4.13 ± 0.30
EtOH treated	4.62 ± 0.26	63.29 ± 6.81	1.79 ± 0.14

[a] Mammary tissue isolated from Control and EtOH treated rats was homogenized in 0.15 M Tris–HCl/1 mM KH_2PO_4 (pH 7.4), centrifuged at 600 × *g* and the supernatants were used to carbonyl determination as described in Section 2. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). $P < 0.05$ (Control vs. EtOH treated).

[b] Mammary tissue isolated from Control and EtOH treated rats was homogenized in 0.15 M Tris–HCl/1mM KH_2PO_4 (pH 7.4), centrifuged at 600 × *g* and the supernatants were used to sulfhydryl determination as described in Section 2. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). $P < 0.005$ (Control vs. EtOH treated).

[c] 8-oxodGuo was measured in DNA extracted from mammary tissue and analyzed by HPLC with electrochemical detector as described in Section 2. All samples were analyzed in triplicate, from groups of five animals each. $P < 0.05$ (Control vs. EtOH treated).

protein sulfhydryl content and in the 8-oxodGuo content of DNA (Table 3). Under similar experimental conditions, no statistically significant increases were observed in the protein carbonyl content in the EtOH treated animals.

3.7. Ultrastructural alterations in the mammary tissue of rats treated with EtOH liquid diet

3.7.1. Control

The secretory cells occurred as a single layer surrounding a central lumen to form the acinus. The mammary gland is composed of an inner continuous layer of columnar or cuboidal epithelial cells and an outer discontinuous layer of myoepithelial cells (Fig. 4a and c). The luminal surface of the epithelial cells had a profusion of microvilli. The nuclear membrane often showed invaginations. Large aggregates of chromatin granules occurred, particularly peripherally. The nucleolus is large. Free ribosomes, scanty profiles of rough endoplasmic reticulum and few mitochondria were found in the cytoplasm. Interdigitations between adjacent epithelial cells were frequent (Fig. 4a and b). The myoepithelial cells had the plasma membrane bordering the basement membrane and some cells show undulating form (Fig. 4c and d).

3.7.2. Ethanol treated

Epithelial cells of mammary gland from animals chronically treated with ethanol showed remarkable differences when compared to Controls. The alveolar cell cytoplasm was vacuolized, nuclear membrane was dilated and large lipid droplets were seen (Fig. 5b and c). Luminar microvilli of ductal epithelial cells were more irregular and fewer in number than in control mammary gland (Fig. 5a and b). Many nuclei show a degree of irregularity of form and increased nucleocytoplasmic exchange. Pores both with and without diaphragms show that material can pass unimpeded (Fig. 5d).

4. Discussion

In agreement with previous reports from our laboratory, the present studies confirm that rat mammary tissue cytosolic and microsomal fractions are able to metabolize EtOH to AC in the presence of adequate cofactors or cosubstrates (Castro et al., 2001, 2003). Interestingly, the results reported here show that both metabolic pathways of EtOH metabolism significantly increased their activity after repetitive alcohol drinking of the standard Lieber and De Carli diet for 28 days. The increased cytosolic pathway of EtOH activation involved the participation of the XOR enzyme as indicated by the needed purine cosubstrate and the inhibition by allopurinol (Castro et al., 2001). The increased microsomal metabolism of EtOH to AC was attributed to a peroxidase-like enzyme, presumably a lipoxygenase because of its response to different inhibitors (Castro et al., 2003). The observed inductive effect of that lipoxygenase activity by repetitive alcohol drinking might be of particular significance. In effect, recent evidence indicates that lipoxygenases and the signaling pathways that are involved in their activation are also important for carcinogenesis and tumor progression (reviewed by Catalano and Procopio, 2005). Several lipoxygenases contributed to the formation of products which are involved in angiogenesis control; serve various inmmunoregulatory functions; control apoptosis and promote cell proliferation (Catalano and Procopio, 2005). However, the type of lipoxygenase involved and the increased presence of those relevant products in the breast of alcohol treated animals remains to be established.

This XOR enhanced activity after chronic alcohol exposure may be of particular significance not only because of the participation of this enzyme in the genera-

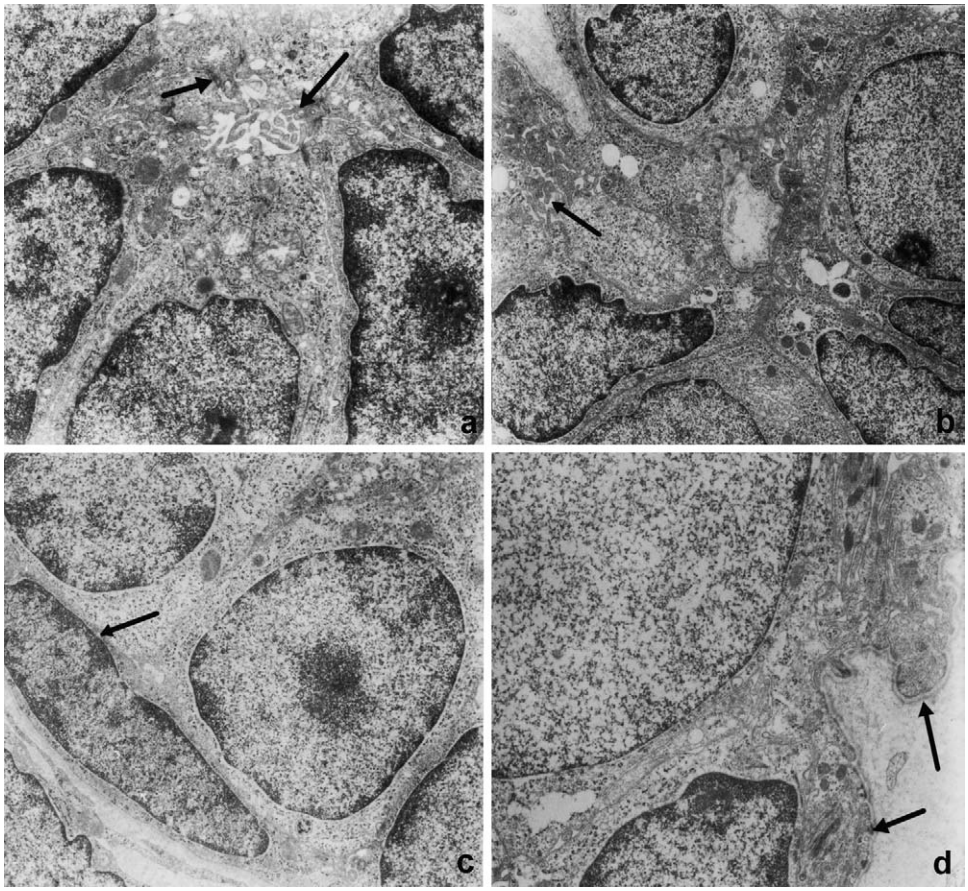

Fig. 4. Electron micrographs of resting rat mammary gland (Control). (a) Epithelial cells. The lumen (arrow) is ringed with microvilli. Large nuclei show chromatin concentrated near the nuclear membrane. The nucleolus is large. Desmosomes connect the epithelial cells near the luminal border (arrow), 11,000×. (b) Epithelial cells. Microvilli are also present on non-luminal surfaces (arrow). Endoplasmic reticulum is widely distributed, 11,000×. (c) Epithelial and mioepithelial cells resting on a thin basal lamina. The mioepithelial cells (arrow) is peripheral to the epithelial cells and tangential to them, 14,080×. (d) The basement membrane is thin (arrows). The myoepithelial cell have hemidesmosomes and small mitochondria. The cellular outline is irregular, 14,080×.

tion of AC from EtOH in breast but also because XOR has been considered a key enzyme in the cell signaling processes and in the generation of reactive oxygen species (ROS) (Harrison, 2004; Hensley et al., 2000). ROS in turn are being considered second messenger molecules involved in the signaling cascade controlling diverse critical cellular events such as proliferation, apoptosis and inflammation (Behrend et al., 2003). One interesting feature of XOR is that it is already present in high amounts in control mammary tissue and that is located in the epithelial cells as shown by our and previous histochemical studies (Jarasch et al., 1981; Kooij et al., 1991). The epithelial cells from terminal end buds are known to be the site of genesis of human mammary tumors (Stewart and Kleihues, 2003).

This enhanced cytosolic and microsomal metabolic production of AC from EtOH might be of significance. The additional contribution of cytosolic alcohol dehydrogenase pathway of AC production may be more limited. On one hand, previous studies by Guerri and Sanchis (1986) evidenced that no ADh activity was found in homogenates of rat mammary tissue. On the other hand, Triano et al. (2003) reported that human mammary tissue contains a class I ADh having a limited potential to biotransform EtOH to AC. Previous studies from other laboratories reported the presence in human mammary tissue of CYP2E1 (Iscan et al., 2001; Kapucuoglu et al., 2003). CYP2E1 presence might also contribute to AC formation in mammary tissue. CYP2E1 is also known to be induced by EtOH drinking and to be important in production of 1-hydroxyethyl radicals and reactive oxygen species (Lieber, 2004). That ability of the CYP2E1 mediated pathway of EtOH bioactivation proved, in the case of liver, to promote a lipid

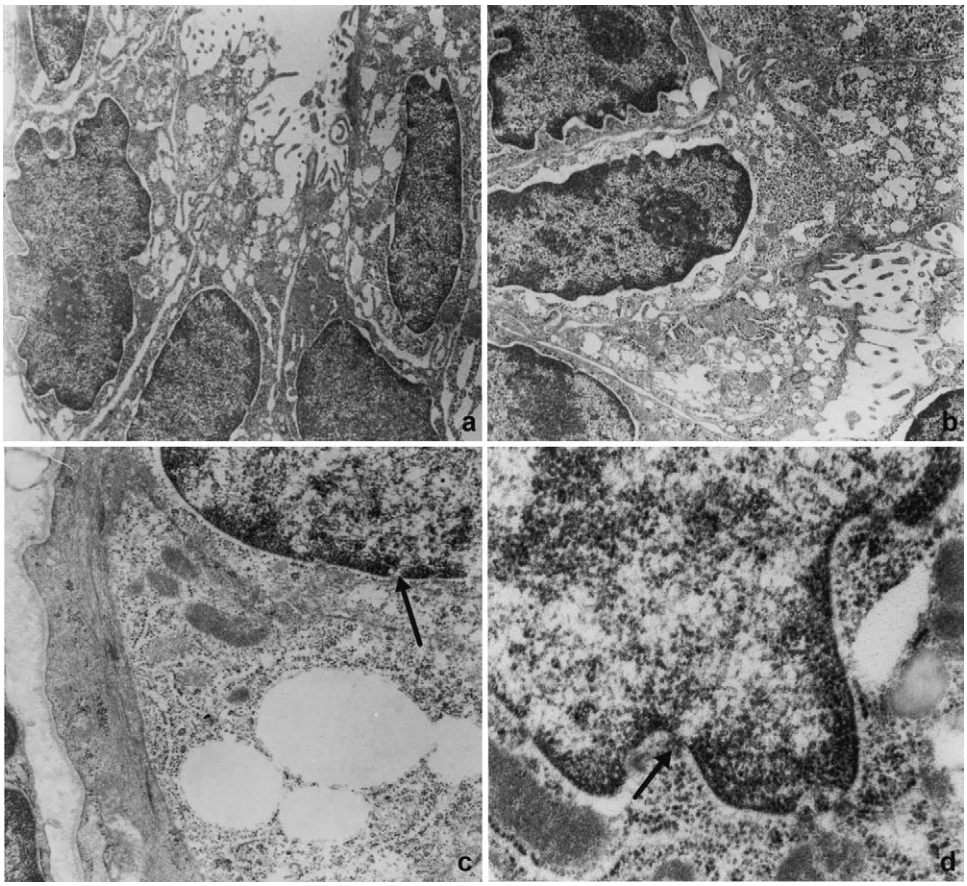

Fig. 5. Electron micrographs of resting rat mammary gland (EtOH treated). (a) Epithelial cells show extended vacuolization, with broken mirovilli in the luminal border and dilated intercellular space, 11,000×. (b) Nuclei have irregular shape and dilated perinuclear membrane, 11,000×. (c) Basement membrane looks hazy and thicker. Cytoplasm shows the presence of large lipid droplets. Numerous nuclear pores (arrow) without diaphragm occur in certain regions of the nuclear membrane, 11,000×. (d) Sometimes these pores channel material to cytoplasm (arrow), 23,760×.

peroxidation process which, after the formation of 4-hydroxynonenal, leads to the generation of highly carcinogenic etheno DNA adducts (Frank et al., 2004). In our hands, however, the participation of P450 mediated metabolism of EtOH to AC in rat breast microsomal fraction was not relevant and of not detectable significance when compared to that of the lipoxygenase mediated pathway (Castro et al., 2003). Furthermore, our laboratory recently made a preliminary observation about an additional pathway of EtOH metabolism to AC present in the mitochondrial fractions which is NADH/NADPH dependent and rotenone insensitive apparently being at the outer mitochondrial membrane (de Castro et al., 2005). That is, the mammary tissue epithelial cells have different alternative pathways to produce AC from EtOH. In contrast, rat mammary tissue has only limited AldDh activity which significantly decreases in chronically alcohol exposed rats (Guerri and Sanchis, 1986). A limited ability to handle AC can make mammary tissue particularly susceptible to AC generation in situ or to the one arriving to it via blood. That behavior might be involved in the already evidenced increased risk to develop alcohol-associated breast cancer in alcohol consumers either with one ADh1C*1 allele encoding for a fast metabolizing form of the ADh1C enzyme (Coutelle et al., 2004) or for the case of premenopausal women having the ADH3 (1–1) genotype (Freudenheim et al., 1999) or among those who carry susceptible glutathione *S*-transferase genotypes (e.g., the GSTT1-nullgenotype) (Zheng et al., 2003). This might be significant considering that AC proved to be carcinogenic, it is a highly mutagenic chemical and is able to interact with many cellular constituents including DNA, proteins, lipids, glutathione, and others (Dellarco, 1988; Lieber, 2004; Pöschl et al., 2004; Woutersen et al., 1984, 1986).

There are other interesting potential consequences of the here reported observations. They concern the possibility that because of the different effects simultane-

ously occurring during repetitive EtOH exposure of the mammary tissue, it became increasingly susceptible to oxidative stressful conditions. In effect, increased XOR and lipoxygenase activity by itself would lead to not only higher generation of ROS but also when occurring in the presence of EtOH, to increased generation of free radicals (Castro et al., 2001). Further, the generation of increased levels of AC may provoke decreases in GSH content as it was repetitively observed in liver (Lieber, 2004). GSH and GSH-dependent enzymes such as GSH peroxidase, GSSG reductase and GSH transferase are a vital first line defense against oxidative stressful conditions (Cook et al., 2004; Hussain et al., 2003).

In the initial observations made in the present studies, we show that this might be the case. For example, in our studies on the *t*-butylhydroperoxide induced chemiluminiscence in rat mammary tissue homogenates we observed that homogenates from Control rats delayed the response in chemiluminscence to the challenging hydroperoxide when compared to those receiving the EtOH containing diet. The obtained results suggest that the mammary tissue from animals exposed to EtOH have diminished defenses against oxidizing challenges (Török, 2004). However, the experiments do not give indication about the nature of the defensive processes involved which are being compromised. Further, they do not show whether in fact any oxidative stress may involve DNA, proteins, lipids or other key molecules. In these initial experiments, we have some evidence that an oxidative stress process has been initiated. In effect, in the rat mammary tissue upon chronic exposure to EtOH, the animals revealed the presence of significantly increased levels of lipid hydroperoxides. Further, the protein sulfhydryl content of mammary tissue proteins decreased in EtOH treated animals. This could be of significance considering that many SH enzymes play a key role in cell functioning and also in cell signaling processes (Liebler and Guengerich, 2005). These findings suggest that oxidative stress processes are taking place. That might be of some significance in light of the well established correlation between oxidative stress and tumor promotion and cancer (Hussain et al., 2003; Cook et al., 2004). Notwithstanding, we failed to observe in our experiments any increase in protein carbonyls or in 8oxodGuo content in the mammary tissue protein or DNA. That could cast some doubts about the relative importance of oxidative stress in the alcohol promoted cancer. We are not able to fully explain at present the heterogeneous response of the different target molecules to the occurring EtOH promoted oxidative stress. One possibility might be that to provoke those oxidative alterations in proteins or DNA, much longer periods of EtOH administration and/or special dietary conditions could be necessary. Other authors reported equivalent observations in experiments on the effects of EtOH in the esophagi of rats (Asami et al., 2000). Still other factors might be involved. It is interesting in this respect, that several authors failed to observe increased levels of 8oxodGuo in DNA from different target tissues other than the mammary one and even in cultured breast epithelial cells exposed to EtOH (Bianchini et al., 2001; Lodovici et al., 2000; Asami et al., 2000; Singletary et al., 2004). Further studies are needed to answer these and other questions.

The in situ metabolic generation of deleterious EtOH metabolites such as AC and 1HEt and the EtOH promoted oxidative stress may also lead to alterations in breast epithelial cells. Our structural studies in mammary tissue from animals repetitively exposed to EtOH show that this is the case (Fig. 5). Of particular significance may be the high degree of irregularity of many nuclei. Irregularity of the nuclear form provides an increased area of contact between the nucleus and the cytoplasm and has been correlated with increased nucleo-cytoplasmic exchanges and heightened metabolic activity. Nuclear irregularities of different intensity and type were previously observed in tumor cells (Ghadially, 1982). The dilatation of the nuclear and endoplasmic reticulum membranes is not an unexpected feature since microsomal fractions of breast tissue are also involved in EtOH metabolism to reactive metabolites (Castro et al., 2003). More important, we are not at present in position to weight the relative contribution of alcohol drinking to increasing estrogen levels (Singletary and Gapstur, 2001) in the ultrastructurally observed alterations or whether both and/other factors play a role. Consequently, all the presently discussed findings should only be considered as a contribution to a working hypothesis which require further experiments to be established.

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