Ethanol and acetaldehyde enhance benzo[a]pyrene–DNA adduct formation in human mammary epithelial cells

Sean L. Barnes, Keith W. Singletary and Randall Frey

Department of Food Science and Human Nutrition, University of Illinois, Urbana, IL 61801, USA

A positive association between alcohol intake and breast cancer risk has been found in many epidemiological studies, yet the biological basis for this association is incompletely understood. The present experiments were designed to determine the chronic effect of physiologically relevant concentrations of ethanol (5–25 mM) and acetaldehyde (0.5–5.0 µM) on benzo[a]pyrene (B[a]P)–DNA adduct formation in the non-neoplastic human mammary epithelial cell line, MCF-10F. We tentatively identified the major adduct formed as the anti-dihydrodiol epoxide of B[a]P bound to deoxyguanosine (dGuo). For these studies, cells were treated with ethanol or acetaldehyde for 6 days before exposure to B[a]P at 0.02 or 0.08 µg/ml. Cells incubated with B[a]P at 0.08 µg/ml for 12 h after 6 days’ exposure to 15 and 25 mM ethanol exhibited 1.3- and 2.2-fold increases in B[a]P–DNA adduct formation, respectively. MCF-10F cells treated with B[a]P at 0.02 µg/ml for 12 h after 6 days’ exposure to acetaldehyde at 0.5–5.0 µM concentrations exhibited increases in adduct formation from 3.4- to 4.8-fold compared with controls. For cells incubated with 0.08 µg/ml B[a]P after 6 days’ exposure to 2.5 and 5.0 µM acetaldehyde, adduct levels increased 1.4- and 1.7-fold, respectively, compared with controls. Treatment of cells with ethanol did not affect the activity of cytochrome P450 1A (ethoxyresorufin O-deethylase), whereas the protein expression of glutathione S-transferase and NAD(P)H-quinone oxidoreductase (GSTP1-1 and QR), as judged by immunoblotting, decreased significantly after ethanol exposure. These studies indicate that ethanol and acetaldehyde treatment of the non-neoplastic human mammary epithelial cell line MCF-10F before addition of the environmental carcinogen B[a]P can lead to increased formation of the anti-BP-dihydrodiol epoxide–dGuo adduct. The influence of ethanol appears to be due, in part, to a decrease in protein expression of the phase II detoxification enzyme GSTP1-1. Thus, this report provides evidence for a biological mechanism whereby alcohol consumption may increase the risk of breast cancer in women.

Breast cancer is a major cause of cancer-related death among women in the USA. Numerous epidemiological studies have reported a positive association between alcohol intake and breast cancer risk among women internationally (1). Recently, a pooled analysis of six cohort studies provided additional confirmation that the risk of breast cancer increases linearly as alcohol consumption increases, from 1 and 5 drinks/day (2). The World Cancer Research Fund and others have described alcohol intake as being one of the most consistent enhancers of breast cancer risk (1,3,4). The biological mechanisms responsible for this increased risk have been only partly characterized. Although ethanol itself is not a carcinogen, it has been suggested that ethanol may enhance carcinogen-induced tumorigenesis by acting at any of a number of possible stages in the process of tumor initiation and promotion (5,6). Such mechanisms may include modulating steroid hormone levels, stimulating oxidative stress and modifying the development and proliferative index of the mammary gland (7–11).

Another possible mechanism may involve the enhancement by ethanol of carcinogen-induced DNA damage in target cell DNA. The formation of carcinogen–DNA adducts is considered to be an important prerequisite for the initiation of chemically induced carcinogenesis (12). In this regard, carcinogen-induced DNA damage resembling that caused by the environmental contaminant and polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) has been reported to be present in human breast tissue (13,14). Although exposure to ethanol may increase the metabolic activation of procarcinogens (15–17) and thereby increase the likelihood of increased carcinogen–DNA binding in various tissues, such effects in human breast cells have not been demonstrated to date. The effect of ethanol on carcinogen bioactivation and detoxification depends on the carcinogen studied, the target tissue, gender and whether ethanol is administered acutely or chronically (18,19). For example, it has been reported that ethanol treatment increases tissue glutathione and glutathione S-transferase (GST) activity in other instances can deplete tissue glutathione, depress GST activity and enhance xenobiotic toxicity (20–25). With this in mind, the effect of ethanol on carcinogen metabolism and ultimate carcinogen–DNA adduct formation needs to be determined for each specific tissue. It is also relevant to examine an influence of acetaldehyde on carcinogen–DNA adduct formation, since acetaldehyde is the first product of ethanol metabolism and a mediator of some of the toxic effects associated with ethanol (26,27). The purpose of these experiments, therefore, was to evaluate the effect of chronic exposure to physiologically relevant concentrations of ethanol and acetaldehyde on B[a]P–DNA adduct formation in MCF-10F cells, a non-neoplastic, spontaneously immortalized human mammary epithelial cell line. These cells behave like normal breast epithelial cells in culture and can be transformed to the neoplastic phenotype by brief exposure to several mammary carcinogens, including B[a]P (28,29). Thus, they can provide an important tool for examining the effects of ethanol and acetaldehyde on events involved in the initiation of mammary carcinogenesis.

The MCF-10F cell line was obtained from the Michigan Cancer Foundation. Cells were cultured at 37°C with 0.5% CO2 as described by Soule et al. (28) and Calaf and Russo (29). The cells (all experiments were from the same passage) were plated in 150 cm2 culture flasks, containing DMEM/F-12 medium (Gibco, Grand Island, NY) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml

Abbreviations: B[a]P, benzo[a]pyrene; dAdo, deoxyadenosine; dGuo, deoxyguanosine; DMBA, 7,12-dimethylbenz[a]anthracene; EROD, ethoxyresorufin O-deethylase; GST, glutathione S-transferase; QR, NAD(P)H-quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon.
amphotericin B, 0.5 μg/ml hydrocortisone, 0.02 μg/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 10 μg/ml insulin and 5% equine serum.

Two studies were performed to characterize B[a]P–DNA adduct formation in MCF-10F cells and to determine the influence of prior exposure to ethanol and acetaldehyde on subsequent B[a]P–DNA adduct formation. To characterize time of exposure and dose of B[a]P on B[a]P–DNA binding, cells (8 × 10^5/150 cm^2 flask) were treated with medium containing final concentrations of 0.02, 0.08 and 0.2 μg/ml B[a]P, each for 6, 12, 24, 48 or 72 h (four flasks/dose/time point). In order to identify tentatively the adduct spots observed, B[a]P–deoxyadenosine (dAdo) and -deoxyguanosine (dGuo) standards were synthesized by reacting dAdo- and dGuo-3′-monophosphates with a racemic mixture of (±)-anti B[a]P dihydrodiol epoxides (Chemsyn Science Laboratories, Lenexa, KN) using the protocol described by Canella et al. (30). To examine the effect of chronic ethanol exposure on B[a]P–DNA adduct formation, cells (1 × 10^5 cells/150 cm^2 flask) were allowed to attach for 48 h. Fresh medium was then added, containing ethanol at concentrations of 0, 5, 15 or 25 mM (eight flasks/treatment group) with fresh medium provided daily. These concentrations of ethanol represent blood ethanol concentrations reported after consumption of low to moderate amounts of alcohol (31,32). After 6 days, media in all groups were removed and replaced with control medium, and cells were allowed to incubate for 2 h. This 2 h period was chosen in order to allow clearance of ethanol so that the effects of acute ethanol exposure would be minimized. After this 2 h period, groups of flasks (four flasks/ethanol dose/B[a]P dose) were provided with fresh medium containing B[a]P at either 0.02 or 0.08 μg/ml. After 12 h the cells were harvested and DNA was isolated. To examine the effect of exposure to acetaldehyde on B[a]P–DNA adduct formation, cells (1 × 10^5 cells/150 cm^2 flask) were allowed to attach for 48 h. Then fresh medium was added containing acetaldehyde at final concentrations of 0, 0.5, 2.5 or 5.0 μM. These doses were chosen based on human data indicating that moderate consumption of alcohol by women can result in blood acetaldehyde concentrations of up to ~5 μM (33). Due to the volatility of acetaldehyde, cells were exposed to fresh acetaldehyde every 12 h. After 6 days, media in all groups were removed and replaced with control medium, and cells were allowed to incubate for an additional 2 h (to minimize the effects of acute acetaldehyde exposure). After this 2 h period, groups of flasks (four flasks/acetaldehyde concentration/B[a]P dose) were provided with control medium containing B[a]P at 0.02 or 0.08 μg/ml. After 12 h of incubation, the cells were harvested and DNA was isolated using a Genomix DNA extraction kit (Tel-Test, Friendswood, TX). All DNA samples exhibited A_{260}/A_{280} ratios of ≥1.8. For {sup}3^{2}P post-labeling of DNA adducts, aliquots of 5 μg of DNA were digested to deoxyribonucleotides-3′-monophosphates, post-labeled and separated by thin layer chromatographic (TLC) procedures described by Singletary et al. (34). After TLC plates had been washed and dried, adduct spots were located by autoradiography and quantified by scintillation counting.

To determine the effect of ethanol on cytochrome P450 1A activity, cells were treated with ethanol at final concentrations of 0, 5 or 25 mM (three flasks/treatment group) for 6 days as described previously. Cells were then harvested and the microsomal fraction was prepared in 0.15 M KCl, 10 mM HEPES–HCl pH 7.6 and 1 mM EDTA in 20% glycerol, and assayed for ethoxyresorufin O-deethylase (EROD) activity using the protocol described by Pohl and Fouts (35).

To determine the effect of ethanol on NAD(P)H-quinone oxidoreductase (QR) and GSTP1-1 by immunoblotting, cells were harvested in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholic acid, 150 mM NaCl, 50 mM Tris pH 7.5, 0.5 mM EDTA, 50 mM NaF, 10 mM NAPG, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 0.02 mg/ml aprotonin, leupeptin and pepstatin. Equal quantities of total protein from each treatment group were loaded on to polyacrylamide gels and separated by SDS–polyacrylamide gel electrophoresis. After membrane transfer, QR protein was located using rabbit anti-NQO1E.coli antibody, generously provided by Dr Su-Shu Pan (University of Pittsburgh School of Medicine, Pittsburgh, PA), and GSTP1-1 protein was located using an antibody from Oxford Biomedical (Oxford, MI). Proteins were located by enhanced chemiluminescence and quantified by densitometry.

Statistical analyses of differences in adduct levels and in enzyme quantities between treatments were examined by analysis of variance (ANOVA) with Fisher’s least-squares difference test for post hoc comparisons. Differences between means were considered statistically significant when P was <0.05.

We observed that exposure of MCF-10F cells to B[a]P resulted in the formation of predominantly one adduct spot. An autoradiogram of a representative B[a]P–DNA adduct sample is depicted in Figure 1a. The B[a]P–DNA adduct formed in MCF-10F cells was tentatively identified as that due to binding of the anti dihydrodiol epoxide of B[a]P predominantly with dGuo. The autoradiogram of adducts resulting from the reaction of the racemic mixture of (±)-anti-B[a]P dihydrodiol epoxides with deoxyadenosine (dAdo) and with dGuo is presented in Figure 1b and c, respectively. The adduct spot location for B[a]P–DNA from MCF-10F cells most closely aligned with that due to the B[a]P–dGuo adduct standard. Also, when the B[a]P–DNA adduct sample from MCF-10F cells was mixed with the synthesized B[a]P–dAdo and B[a]P–dGuo standards, it co-migrated with the B[a]P–dGuo standard but not with that due to binding of B[a]P with

Fig. 1. Autoradiograms of B[a]P–DNA adduct spots (a) from MCF-10F cells after B[a]P exposure, (b) from synthesized B[a]P-dAdo and (c) B[a]P-dGuo adducts and (d and e) from mixtures of each standard with MCF-10F DNA. OR, origin.
dAdo (Figure 1d and e). The formation of DNA adducts when MCF-10F cells are exposed to three concentrations of B[a]P for time periods ranging from 6 to 72 h is shown in Figure 2. Maximum B[a]P–DNA adduct formation occurred 12 h after B[a]P exposure for the groups treated with 0.02 and 0.08 μg/ml B[a]P. For the 0.2 μg/ml dose, peak adduct formation occurred 24 h after B[a]P treatment. B[a]P–DNA adduct formation for the groups treated with 0.08 or 0.2 μg/ml was significantly higher than that from the group treated with 0.02 μg/ml at all time points. The relative capacity of MCF-10F cells to form DNA adducts after exposure to similar concentrations of B[a]P and the polycyclic aromatic hydrocarbon (PAH) 7,12-dimethylbenz[a]anthracene (DMBA) also was assessed. B[a]P–DNA adduct formation was significantly greater than total DMBA–DNA adduct formation when evaluated at the 0.08 μg/ml (0.32 μM B[a]P and 0.31 μM DMBA) and 0.2 μg/ml (0.79 μM B[a]P and 0.78 μM DMBA) doses (Figure 3). The influence of exposure to B[a]P alone on the activities of EROD, GST and QR in cultured MCF-10F cells was also assessed. No statistically significant changes in the activities of these enzymes were observed after incubation of cells for 12 h with 0.02 or 0.08 μg/ml B[a]P. Treatment of cells with B[a]P at concentrations of 0, 0.02 and 0.08 μg/ml resulted in mean ± SD EROD activities of 85.5 ± 13.2, 98.1 ± 1.0 and 83.8 ± 20.6 pmol/mg protein, respectively (P > 0.05). Treatment of cells with B[a]P at 0, 0.02 and 0.08 μg/ml was associated with QR activities of 190.5 ± 28.8, 113.7 ± 13.7 and 125.8 ± 36.8 nmol/min/mg protein, respectively, and was associated with GST activities of 243.4 ± 27.4, 166.5 ± 12.7 and 168.3 ± 19.9 nmol/min/mg protein, respectively (P > 0.05).

The effects of incubating MCF-10F cells with three concentrations of ethanol for 6 days before treatment with either of two doses of B[a]P are presented in Figure 4. The viability of cells as assessed by trypan blue exclusion was >95% at all ethanol concentrations examined. Ethanol concentrations of 5–25 mM were chosen to represent blood ethanol concentrations that might result from low to moderate alcohol consumption. We also wanted to measure the response to ethanol at what could be considered low and intermediate doses of B[a]P, namely 0.02 and 0.08 μg/ml, respectively. For cells treated with the low B[a]P dose, a trend towards increased B[a]P–DNA adduct formation was observed among all the ethanol-treated groups. However, the adduct level was marginally significant (P = 0.054) only for the 15 mM ethanol group. When MCF-10F cells were treated with the higher B[a]P dose, a significant 1.3- and 2.2-fold increase in B[a]P–DNA adduct levels was observed for the 15 and 25 mM ethanol groups, respectively, compared with controls. Exposure of MCF-10F cells to physiologically relevant concentrations of acetaldehyde also affected B[a]P–DNA adduct formation (Figure 5). For cells treated with the low B[a]P dose, statistically significant 3.4-, 4.0- and 4.8-fold increases in B[a]P–DNA adduct levels were observed at acetaldehyde concentrations of 0.5, 2.5 and 5.0 μM acetaldehyde treatment.
5.0 µM, respectively, compared with controls. At the higher dose of [a]P, significant 1.4- and 1.7-fold increases in [a]P–DNA adduct formation were observed for cells treated with acetaldehyde at 2.5 and 5.0 µM, respectively, compared with controls.

In order to determine a possible mechanism for the enhancement by ethanol of [a]P–DNA adduct formation, the effects of ethanol concentration on cytochrome P450 1A activity (EROD) and on the protein expression of the phase II detoxification enzymes QR and GST were measured. GSTP1 was chosen because it is involved in conjugation/detoxification of [a]P diol epoxide and in epidemiological studies, allelic variants have been linked to cancer susceptibility (36,37). No significant effect of ethanol on EROD activity was observed. For cells treated with 0, 5 and 25 mM ethanol, EROD activity was (mean ± SEM) 173.8 ± 7.1, 173.2 ± 1.9 and 187.5 ± 3.7 pmol/min/mg protein, respectively. GSTP1-1 protein expression, as determined by western blotting, was influenced by treatment with ethanol (Figure 6a). GSTP1-1 protein content of cells decreased by 20%, 73% and 32% for MCF-10F cells treated with 5, 15 and 25 mM ethanol, respectively, compared with controls. QR protein expression also was lower for cells treated with ethanol, but the difference was not significant (P = 0.06) (Figure 6b).

The present studies provide evidence that the human mammary epithelial cell line MCF-10F is capable of metabolizing [a]P to anti-[a]P-dihydrodiol epoxide-dGuo adducts. In MCF-10F cells exposed to [a]P, the formation of [a]P–DNA adducts increased in a dose- and time-dependent manner. In addition, the MCF-10F cells were more effective at forming [a]P–DNA adducts compared with DMBA–DNA adducts at the concentrations of [a]P and DMBA examined. These results are consistent with those of Moore et al. (38), who reported that primary human mammary cells metabolize [a]P to DNA-binding forms, and that DNA binding due to [a]P is greater than that from treatment with DMBA. This indicates that MCF-10F cells, although differing from primary cells in being immortalized, still retain the same capacity as primary mammary epithelial cells to metabolize [a]P more readily to DNA-binding forms compared with that for DMBA. In addition, DMBA requires the combined actions of cytochrome P450 1A1 and 1B1 for conversion into a diol epoxide, with the latter enzyme probably being present at very low levels in MCF-10F cells (39). Also, DMBA is a poor ligand of the aryl hydrocarbon receptor and thus would be a poor inducer of cytochrome P450 1A1 and 1B1. The present study also is consistent with a report by Stampfer et al. (40), in which binding of [a]P to dGuo was the major adduct formed in primary human mammary epithelial cells exposed to [a]P.

The present studies also indicate that chronic exposure of MCF-10F cells to ethanol and acetaldehyde can lead to enhanced [a]P–DNA adduct formation. The enhancing effect of ethanol on [a]P–DNA adduct formation was most pronounced for cells exposed to the higher dose of [a]P (0.08 µg/ml), although a trend toward increased adducts was observed for cells treated with [a]P at 0.02 µg/ml. In contrast, chronic exposure of MCF-10F cells to acetaldehyde resulted in increased [a]P–DNA adduct formation at both doses of [a]P examined. At the lower dose of [a]P, all three concentrations of acetaldehyde enhanced adduct formation, whereas at the higher [a]P dose, only 2.5 and 5.0 µM acetaldehyde increased formation, compared with controls. Thus, the magnitude of the increase in [a]P–DNA adduct formation due to ethanol or acetaldehyde treatment is dependent on both the concentration of ethanol or acetaldehyde and the dose of the carcinogen to which the cells are exposed. These enhancing effects of ethanol and acetaldehyde on [a]P–DNA adduct formation were observed in response to physiologically relevant doses of ethanol and acetaldehyde. The ranges of ethanol and acetaldehyde concentrations used in these experiments were derived from reported blood concentrations that have been detected in women who consumed ethanol. For instance, it has been determined that a 120 lb (54 kg) woman who consumes one to three drinks [0.5 oz (14.2 g) ethanol/drink] will achieve blood alcohol concentrations ranging from 5 to 24 mM (31). Consistent with this is a report that women who customarily drank ≤10 g (about one standard drink) of ethanol...
per day exhibited a peak blood alcohol concentration in the lower end of the range (5 mM), while those who customarily consumed 40–60 g of ethanol exhibited peak blood alcohol concentrations closer to 25 mM (32). With regard to blood acetaldehyde concentrations, women consuming ethanol at doses of 0.34–1.02 g/kg body weight exhibited blood acetaldehyde concentrations of ≈6 µM, with a mean of 2 µM (33). It appears, therefore, that exposure of MCF-10F cells to daily doses of ethanol and its primary metabolite, acetaldehyde, at concentrations attainable from human alcohol consumption, is associated with an increase in B[a]P-DNA binding.

This increase in binding of B[a]P to DNA after ethanol treatment may in part be due to ethanol-associated decreases in protein expression of the phase II detoxification enzyme GST. Although GSTP1-1 protein expression decreased significantly for cells treated with 15 or 25 mM ethanol compared with controls, the decline in protein expression was not strictly dose-dependent. The reason for this decrease in GSTP1-1 protein in response to ethanol is not known. It has been reported for other cells that ethanol exposure is associated with reduced mRNA, destabilization of mRNA or alteration of signal transduction pathways that regulate gene expression or of post-translational processing (41–45). Ethanol also has been observed to alter protein synthesis and degradation in numerous tissues (46–48). It is possible that ethanol and/or acetaldehyde may inhibit adduct repair. Although we observed no significant increase in cytochrome P450 1A activity after ethanol treatment, it is possible that ethanol may affect the more recently identified P450 1B, an enzyme identified as playing a substantial role in the bioactivation of PAH by extrahepatic tissues (49). The mechanism(s) whereby ethanol exposure leads to the decreased expression of GSTP1-1 in nonneoplastic human mammary epithelial cells and whether acetaldehyde treatment also leads to similar responses in GST and QR protein expression warrants further examination.

In summary, the nonneoplastic human epithelial cell line MCF-10F metabolizes B[a]P predominantly to a B[a]P-DNA adduct tentatively identified as an anti-B[a]P-dihydrodiol epoxide–dGuo adduct. When cells are exposed to physiologically-relevant concentrations of either ethanol or acetaldehyde prior to dosing with B[a]P, adduct formation is increased. This enhancement of B[a]P-DNA adduct formation by ethanol in part may be explained by reduced GSTP1-1 protein expressed in the ethanol-treated cells. These results provide evidence that a possible mechanism by which alcohol intake may be enhancing breast cancer risk in humans is through an ethanol- and acetaldehyde-associated increase in carcinogen-DNA adducts in the target mammary epithelial cells. The mechanisms for this action of ethanol and acetaldehyde warrant further study.

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