



CYP1A1 and CYP1B1 gene expression and DNA adduct formation in normal human mammary epithelial cells exposed to benzo[a]pyrene in the absence or presence of chlorophyllin

Kaarthik John^{a,b}, Rao L. Divi^c, Channa Keshava^b, Christine C. Orozco^c, Marie E. Schockley^c, Diana L. Richardson^b, Miriam C. Poirier^c, Joginder Nath^a, Ainsley Weston^{a,b,*}

^a Genetics and Developmental Biology Program, West Virginia University, Morgantown, WV, United States

^b Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, CDC, Morgantown, WV, United States

^c Carcinogen-DNA Interactions Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, United States

ARTICLE INFO

Article history:

Received 23 October 2009

Received in revised form 9 December 2009

Accepted 11 December 2009

Keywords:

Real-time-PCR

BPDE-DNA chemiluminescence

immunoassay

Chemoprevention

Chlorophyllin

Polycyclic aromatic hydrocarbons

DNA adducts

ABSTRACT

Benzo[a]pyrene (BP) is a potent pro-carcinogen and ubiquitous environmental pollutant. Here, we examined the induction and modulation of CYP1A1 and CYP1B1 and 10-(deoxyguanosin-N²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPdG) adduct formation in DNA from 20 primary normal human mammary epithelial cell (NHMEC) strains exposed to BP (4 μM) in the absence or presence of chlorophyllin (5 μM). Real-time polymerase chain reaction (RT-PCR) analysis revealed strong induction of both CYP1A1 and CYP1B1 by BP, with high levels of inter-individual variability. Variable BPdG formation was found in all strains by r7, t8-dihydroxy-t-9, 10 epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE)-DNA chemiluminescence assay (CIA). Chlorophyllin mitigated BP-induced CYP1A1 and CYP1B1 gene expression in all 20 strains when administered with BP. Chlorophyllin, administered prior to BP-exposure, mitigated CYP1A1 expression in 18/20 NHMEC strains ($p < 0.005$) and CYP1B1 expression in 17/20 NHMEC strains ($p < 0.005$). Maximum percent reductions of CYP1A1 and CYP1B1 gene expression and BPdG adduct formation were observed when cells were pre-dosed with chlorophyllin followed by administration of the carcinogen with chlorophyllin ($p < 0.005$ for CYP1A1 and CYP1B1 expression and $p < 0.0005$ for BPdG adducts). Therefore, chlorophyllin is likely to be a good chemoprotective agent for a large proportion of the human population.

© 2010 Published by Elsevier Ireland Ltd.

1. Introduction

Humans are constantly exposed to an array of potentially genotoxic xenobiotics, either through the environment, lifestyle or diet. Benzo[a]pyrene (BP), a polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmen-

tal pollutant formed from the incomplete combustion of fossil fuels, food (notably char-broiled meat and fish), tobacco, and other vegetable material [1]. Exposure can occur through ingestion, inhalation or dermal absorption.

BP, a procarcinogen, is bioactivated by BP inducible cytochrome P450s (CYP1A1 and CYP1B1) to reactive, DNA binding electrophiles [2]. Among the various metabolites formed, r7, t8-dihydroxy-t-9, 10 epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) is highly reactive and capable of binding to deoxyguanosine in DNA to form 10-(deoxyguanosin-N²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPdG) adducts [3].

* Corresponding author. Address: NIOSH – CDC, MS-H2900, 1095 Willowdale Road, Morgantown, WV 26505, United States. Tel.: +1 304 285 6221; fax: +1 301 285 5708.

E-mail address: agw8@cdc.gov (A. Weston).

Given the ubiquitous nature of PAHs, complete avoidance of exposure is impossible, thus creating a need for intervention strategies to reduce genotoxicity. Chemoprevention, involving the selective modulation of metabolic enzyme pathways, may help to reduce carcinogenic risk. Chlorophyllin, a water soluble copper–sodium metalloporphyrin, derived by treatment of a methanolic solution of the natural pigment chlorophyll with sodium hydroxide or potassium hydroxide [4], is currently used as a food colorant and deodorizer. It has been found to be a safe and effective chemopreventive agent when used in human populations in Africa and China which ingest aflatoxins and are therefore at risk of hepatocarcinoma induction [5,6]. Given its multiple modes of action, which include its role as a cell cycle modulator, Phase II enzyme inducer, antioxidant, carcinogen transport modulator, interceptor molecule and a modulator of Phase I inducers [7–9], we hypothesized that chlorophyllin might be a useful chemopreventive agent for a broad spectrum of the human population.

Normal human mammary epithelial cells (NHMECs) have previously been used as a model for human inter-individual variability in studies examining the metabolism and activation of BP [10]. Normal human breast tissue taken at reduction mammoplasty was used to establish the panel of 20 cell strains used here. In these experiments we examined the induction and inter-individual variability of *CYP1A1* and *CYP1B1* expression, and BpDg adduct formation upon exposure of NHMECs to BP, in the absence or presence of the chemopreventive agent chlorophyllin.

2. Materials and methods

2.1. Cell strains

Primary NHMEC strains were developed from breast tissues discarded at reduction mammoplasty and obtained through the Cooperative Human Tissue Network (CHTN sponsored by the National Cancer Institute (NCI) and National Disease Research Interchange). The isolation of mammary epithelial cells from breast tissue involved a combination of mechanical disruption and enzymatic digestion and has been described in detail elsewhere [11]. The National Institute for Occupational Safety and Health (NIOSH) Human Studies Review Board (HSRB) was informed about this work, but since no personally identifiable data were collected it was not judged to be in the purview of the HSRB. Information on age and ethnicity of donors, as well as the corresponding deidentified pathology report, were provided by the CHTN.

2.2. Chemicals, media and NHMEC exposures

BP was obtained from the NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). Chlorophyllin was purchased from Sigma–Aldrich (St. Louis, MO).

Serum free mammary epithelial basal medium (MEBM medium) was purchased from Clonetics™ (Walkersville, MD). Cells, maintained at 37 °C, 5% CO₂ and 95% relative humidity, were grown in MEBM supplemented with SingleQuots™ (gentamycin sulfate–amphotericin B, recombinant human epidermal growth factor, bovine insulin, bovine pituitary extract and hydrocortisone) according to the manufacturer's instructions. All treatments were carried out using 70% confluent cells at passage 6.

The exposure regimens used were as follows: T1–BP (4 μM) for 24 h; T2–BP (4 μM) plus chlorophyllin (5 μM) co-treatment for 24 h; T3–24 h pre-treatment with chlorophyllin (5 μM) followed by BP (4 μM) for 24 h; and T4–24 h pre-treatment with chlorophyllin (5 μM) followed co-treatment for 24 h with chlorophyllin plus BP (4 μM). In preliminary studies chlorophyllin alone (5 μM) was used in addition to the above treatments. All exposures were carried out in duplicate.

2.3. Expression analysis of *CYP1A1* and *CYP1B1*

Total RNA isolated from all treatment groups (RNeasy kit, Qiagen, Valencia, CA) and 1 μg was reverse transcribed to single stranded cDNA (Advantage RT for PCR kit, BD Biosciences, Palo Alto, CA). Transcribed RNA was used for the quantitation of *CYP1A1* and *CYP1B1* gene expression using an ABI7700 Sequence Detection System, and Taqman® Gene Expression Assays, with Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The expression levels of *CYP1A1* and *CYP1B1* were normalized to that of *GAPDH* and the fold-change expressed using the $2^{-\Delta\Delta CT}$ method [12].

2.4. DNA isolation

Following exposure, adherent cells at ~90% confluency were washed once with 1× PBS and incubated in lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml Proteinase K) at 37 °C for 3 h. DNA from the lysed cells was precipitated directly, using an equal volume of isopropanol, washed twice with 70% ethanol, and dissolved in an appropriate volume of nuclease free water [13].

2.5. BpDg adduct analysis by chemiluminescence immunoassay (CIA)

Antiserum elicited against DNA modified with BPDE [14] was used to measure BpDg adducts in isolated DNA by competitive BPDE–DNA CIA. Briefly, 96-well high-binding LIA plates (Greiner Labortechnik, FRG) coated for 48 h with 1 fmol of sonicated BPDE–DNA (modified to about 1.0%), or calf thymus DNA, were blocked of non-specific binding by incubation with 0.25% casein in phosphate buffered saline containing 0.05% Tween-20 (PBST) for 60 min. Subsequently, serially diluted, heat denatured (95 °C, 4 min) BPDE–DNA standard (1.0 BpDg adduct/10⁶ nucleotides), or sample DNA from treatment groups, were mixed with an equal volume of rabbit anti-BPDE–DNA antibody (diluted to 1:3,000,000 in 0.25% casein), incubated for 15 min at 37 °C, and added to the LIA plate wells. The final mixture was incubated at 37 °C for 90 min. At the end of the incubation, the plates were washed with PBST, incubated with biotinylated anti-rabbit IgG (1:3000 dilution in 0.25% casein in PBST) for 90 min at room temperature and washed again with PBST. The plates were then incubated for 60 min with streptavidin alkaline phosphatase (diluted 1:3000 using 0.25% casein in PBST), washed again with PBST and Tris buffer, and finally incubated with CDP-Star containing Emerald II enhancer (4 °C, overnight). The luminescence was read at 542 nm both at the end of the incubation and on the following day. The limit of detection was 0.3 adducts/10⁹ nucleotides.

2.6. Statistical analysis

Pearson correlation coefficients were used to compare the effects of BP and chlorophyllin treatments in the Excel program [15]. Paired, 2-tailed Student's *t* test was used to compare the adducts formed in the T1 group to the adducts formed in the T2, T3 and T4 groups.

3. Results

3.1. *CYP1A1* and *CYP1B1* gene expression in NHMECs exposed to BP

The NHMEC strains used in this study, derived from 20 donors between 18 and 51 years of age whose pathology reports all indicated normal breast tissues, were exposed (as described in Section 2.2) and examined by RT-PCR for expression of *CYP1A1* and *CYP1B1*. Preliminary studies with exposure to chlorophyllin alone showed no change in the expression of *CYP1A1* and *CYP1B1* (data not shown). In cells exposed to BP alone (T1 group), induction of *CYP1A1* was 3.6- to 95.8-fold (Table 1), and induction of *CYP1B1* was 3.5- to 43.4-fold (Table 2). *CYP1A1* induction was moderately, but significantly, correlated with *CYP1B1* induction

Table 1

CYP1A1 induction (fold-change measured by RT-PCR) in NHMEC strains by BP in the absence (T1^a) and presence (T2, T3, T4) of chlorophyllin.

Cell Strain	T1	T2	T3	T4
M98014	4.6 ± 1.5 ^b	2.8 ± 0.3	8.3 ± 1.9	5.9 ± 0.9
M98015	50.0 ± 6.6	44.8 ± 12.1	42.6 ± 10.5	47.4 ± 9.4
M98011	5.4 ± 2.0	5.1 ± 1.0	5.5 ± 0.6	5.0 ± 2.6
M99003	14.3 ± 5.0	10.2 ± 2.3	11.5 ± 1.6	13.0 ± 4.0
M99015	35.9 ± 4.7	20.8 ± 3.0	31.8 ± 2.7	29.9 ± 9.7
M98019	3.6 ± 1.0	1.9 ± 0.5	3.0 ± 0.6	2.4 ± 0.8
M98025	11.2 ± 3.2	6.0 ± 2.6	9.8 ± 1.6	6.9 ± 0.7
M99021	5.9 ± 1.5	3.7 ± 1.6	3.8 ± 0.4	3.6 ± 0.5
M00012	17.1 ± 2.9	11.0 ± 1.7	12.2 ± 2.8	9.8 ± 2.7
M98026	52.7 ± 12.9	39.0 ± 10.2	31.3 ± 11.5	30.3 ± 6.0
M99004	20.3 ± 2.1	10.8 ± 4.0	10.5 ± 3.4	11.6 ± 1.6
M99016	95.8 ± 9.9	62.4 ± 20.8	62.4 ± 4.8	53.5 ± 5.4
M99006	6.5 ± 1.5	4.2 ± 1.0	5.3 ± 0.6	3.5 ± 0.8
M00004	7.1 ± 2.5	4.0 ± 0.5	4.4 ± 0.8	3.8 ± 1.4
M98035	13.8 ± 2.9	12.8 ± 2.0	9.7 ± 1.9	5.3 ± 1.6
M98030	44.3 ± 12.4	18.5 ± 1.3	25.5 ± 2.1	16.0 ± 4.1
M99005	13.7 ± 1.4	7.9 ± 1.7	8.1 ± 1.1	4.1 ± 1.4
M99025	6.6 ± 2.9	4.1 ± 1.6	2.6 ± 0.5	1.7 ± 0.5
M98016	32.5 ± 3.4	10.0 ± 2.3	13.2 ± 1.4	6.1 ± 2.0
M98013	66.7 ± 12.1	16.4 ± 20.8	14.7 ± 6.9	8.0 ± 5.0

^a T = Treatment groups as defined in Section 2.

^b Numbers represent fold-change ±SD (*n* = 2).

Table 2

CYP1B1 induction (fold-change measured by RT-PCR) in NHMEC strains by BP in the absence (T1^a) and presence (T2, T3, T4) of chlorophyllin.

Cell Strain	T1 ^a	T2	T3	T4
M98014	3.5 ± 0.6 ^b	2.9 ± 0.9	7.9 ± 1.0	6.8 ± 1.8
M98015	43.4 ± 4.8	35.9 ± 3.3	39.7 ± 9.3	39.3 ± 6.9
M98011	4.7 ± 0.9	3.7 ± 1.5	5.7 ± 1.5	6.3 ± 1.5
M99003	19.7 ± 1.9	13.0 ± 1.2	15.3 ± 1.0	16.5 ± 5.2
M99015	33.9 ± 1.7	13.5 ± 2.3	22.6 ± 11.2	19.9 ± 3.7
M98019	6.1 ± 0.4	4.4 ± 0.4	7.9 ± 1.0	6.8 ± 1.8
M98025	8.4 ± 2.2	4.4 ± 1.8	4.8 ± 1.5	3.9 ± 0.5
M99021	8.8 ± 1.4	5.8 ± 1.0	6.3 ± 1.2	5.5 ± 0.9
M00012	17.5 ± 3.1	10.3 ± 1.7	14.6 ± 4.4	11.9 ± 3.3
M98026	31.7 ± 3.3	21.1 ± 1.7	24.2 ± 8.4	23.6 ± 6.4
M99004	12.1 ± 1.3	7.7 ± 1.3	7.0 ± 0.7	6.9 ± 0.8
M99016	8.9 ± 1.4	8.6 ± 1.6	8.3 ± 1.1	6.9 ± 1.3
M99006	8.9 ± 1.6	5.5 ± 0.4	9.9 ± 3.6	4.8 ± 0.8
M00004	7.9 ± 2.6	5.1 ± 0.5	7.3 ± 2.9	4.5 ± 2.0
M98035	9.5 ± 1.9	8.0 ± 1.8	7.3 ± 1.8	3.3 ± 0.4
M98030	16.0 ± 4.1	7.0 ± 1.4	6.5 ± 1.0	4.9 ± 1.8
M99005	9.2 ± 1.8	7.5 ± 0.4	5.8 ± 1.7	3.5 ± 1.3
M99025	22.9 ± 3.9	11.4 ± 3.1	9.2 ± 1.2	6.5 ± 1.3
M98016	14.7 ± 3.0	6.2 ± 0.6	10.0 ± 5.9	3.9 ± 1.1
M98013	42.0 ± 6.9	14.0 ± 1.5	11.5 ± 1.9	7.0 ± 3.2

^a T = Treatment groups as defined in Section 2.

^b Numbers represent fold-change ±SD (*n* = 2).

(*r* = 0.530, *p* < 0.01), replicating data observed previously [12].

In all cells treated with chlorophyllin (exposure groups T2, T3, T4) in addition to BP, there were generalized reductions in induction of both *CYP1A1* and *CYP1B1* (Tables 1 and 2, respectively), compared to the T1 group. Of the three different chlorophyllin treatment regimens, T4 was most effective in mitigating induction of both cytochrome P450s. Induction of *CYP1A1* was reduced between 5.1% and 87.9% by chlorophyllin, with the exception of strain M98014, in which induction was increased by 29.1% in the presence of chlorophyllin (Fig. 1A). Similarly, *CYP1B1*

induction was generally reduced (9.6–83.3%), although it was increased by 11.2% and 33.3% in cell strains M98019 and M98011, respectively (Fig. 1B). Fig. 2 shows a high degree of correlation in the chlorophyllin-mediated reduction of *CYP1A1* and *CYP1B1* induction by BP (*r* = 0.657, *p* = 0.0013).

3.2. DNA adduct levels induced by BP

In NHMEC strains exposed to BP alone (T1 group), BPdG adducts were measured by BPdE-DNA CIA and ranged from 2.5 to 57.5 adducts/10⁸ nucleotides (Table 3). The addition of chlorophyllin resulted in reduced BPdG adduct levels in all three groups (T2, T3, and T4) for all 20 of the NHMEC strains studied, with the greatest decrease in the T4 group (see Table 3 and Fig. 3). Mitigation of BPdG adduct levels was by 25–87% to 1.3–35.4 adducts/10⁸ nucleotides in the T4 group (see last column in Table 3). There was a wide inter-individual variation in response to chlorophyllin, and the 20 cell strains could be divided into three groups: 10 cell strains showing adduct level reductions of 25–50%, 6 cell strains showing adduct level reductions of 50–75%; and, 4 cell strains showing adduct reductions of >75% (Fig. 3).

3.3. Correlations between CYP450 expression and BPdG adducts

In this study, in cells exposed to BP alone (T1 group), neither *CYP1A1* nor *CYP1B1* expression correlated with BPdG adduct formation (*r* = 0.437 for *CYP1A1* and *r* = 0.306 for *CYP1B1*, *p* > 0.10 for both). This result has been observed previously [12]. In addition, in cells exposed to BP with chlorophyllin (T4 group) there were poor correlations for the percentage reduction of BP-induced cytochrome P450 induction with the corresponding percentage reduction in BPdG adduct levels (*r* = 0.249, *p* = 0.096 for *CYP1A1*; and *r* = 0.409, *p* = 0.070 for *CYP1B1*). Similar trends were observed for the other chlorophyllin treatments as well (data not shown). There were good correlations between the numbers of adducts formed by comparison of cells treated with BP alone (T1 group) with cells treated in the presence of chlorophyllin [*r* = 0.947, *p* = 0.0008 for T1 vs. T2; *r* = 0.945, *p* = 0.0017 for T1 vs. T3 and *r* = 0.826, *p* = 0.0004 for T1 vs. T4].

Overall, the cell strains showed wide inter-individual variability in *CYP1A1* and *CYP1B1* induction, BPdG adduct formation, reduction in *CYP1A1* and *CYP1B1* induction by chlorophyllin treatment and mitigation of BP-DNA adduct levels by chlorophyllin treatment. Fig. 4 depicts the consistent pattern of reduction in percent *CYP1A1* and *CYP1B1* gene expression and BPdG adduct formation across most of the 20 NHMEC strains in response to chlorophyllin treatment in group T4 (*p* < 0.005, *p* < 0.005 and *p* < 0.0005 for *CYP1A1*, *CYP1B1* and BPdG adducts, respectively).

4. Discussion

In this study we found that exposure of 20 NHMEC strains, from 20 individual donors, to BP resulted in up-

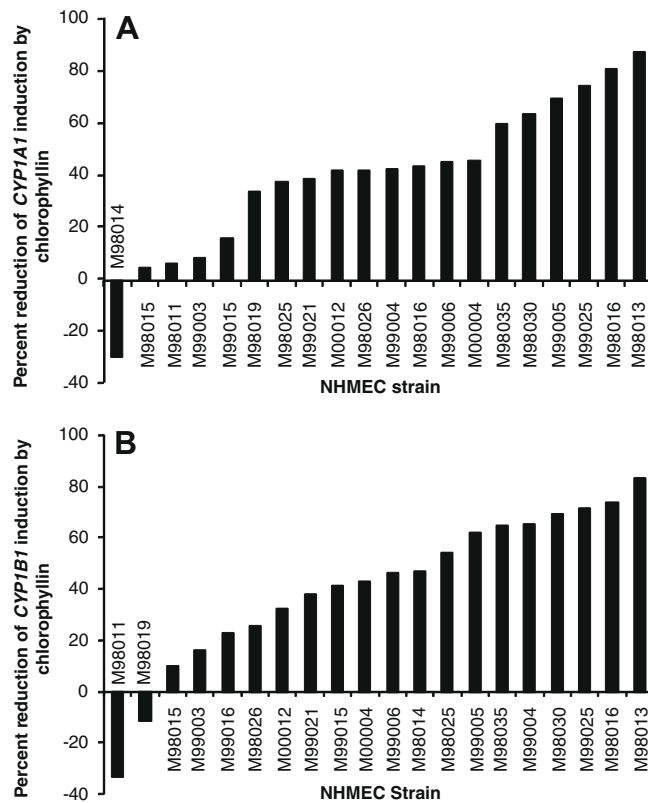


Fig. 1. (A and B) Bar graphs showing percent reduction of expression (by RT-PCR) of *CYP1A1* (A) and *CYP1B1* (B) in each primary NHMEC strain when treatment group T1 (BP alone) was compared with treatment group T4 (24 h chlorophyllin followed by 24 h of BP plus chlorophyllin). The magnitude of the bar represents the extent to which chlorophyllin reduced the BP-induced gene expression.

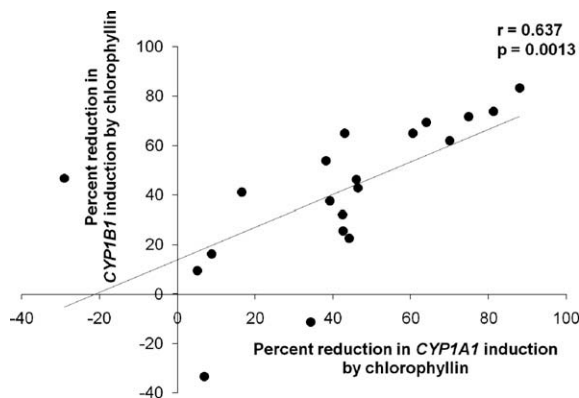


Fig. 2. Strain-wise correlation for the percent reduction in expression of *CYP1A1* and *CYP1B1* in T4 cells compared to T1 cells ($r = 0.637$, $p = 0.0013$); the graph shows data for all 20 NHMEC strains.

regulation of *CYP1A1* and *CYP1B1* in almost all of the strains, and produced measurable BPdG adducts in all of the strains. The addition of chlorophyllin resulted in lowering almost all the BP-induced increases in *CYP1A1* and *CYP1B1* expression, and was effective in reducing BPdG levels in all 20 NHMEC strains. The lack of any exceptions to the chlorophyllin-modulated reduction in DNA damage suggests that chlorophyllin may be a useful chemopreven-

tive agent for the human population because most individuals should respond to chlorophyllin treatment with decreased PAH activation although the magnitude may vary.

The gene expression experiments presented here expand our previous work using DNA microarrays in a single NHMEC strain, M98013 [16]. M98013 was used in this study and values for BP-induced *CYP1A1* and *CYP1B1* expression were similar to those found previously [16], however, the addition of 19 new NHMEC strains revealed considerable variation in levels of BP-induced *CYP1A1* and *CYP1B1* expression. This wide inter-individual variation might be explained, at least in part by polymorphisms in the genes coding for cytochrome P450s and other Phase I enzymes, as well as genes coding for Phase II or detoxication enzymes and DNA repair functions [11]. Other factors may also be relevant. For example, we found a poor correlation between *CYP1* gene expression and donor age (data not shown), in agreement with a previous study showing age-independent variation in *CYP1A1* expression in normal breast tissue samples ($n = 58$) obtained from breast cancer patients as well as individuals with no diagnosis of cancer [17]. In our study, the magnitude of *CYP1B1* induction was much less than that found for *CYP1A1*. *CYP1B1* is a dioxin inducible cytochrome P450 isoform that expressed constitutively in various extrahepatic tissues, including the

Table 3
BPdG adducts formed in NMECs exposed to BP in the absence (T1^a) or presence (T2, T3, T4) of chlorophyllin.

Cell Strain	T1	T2	T3	T4	% Reduction T1 vs. T4 ^c
M98014	4.7 ± 0.6 ^b	2.2 ± 0.4	2.9 ± 0.2	2.1 ± 0.7	55.1 ^c
M98015	8.3 ± 5.0	5.5 ± 3.3	5.5 ± 4.0	4.3 ± 4.1	48.2
M98011	20.8 ± 13.0	15.3 ± 13.1	17.8 ± 9.2	11.7 ± 9.3	43.7
M99003	7.3 ± 1.5	3.3 ± 0.3	3.5 ± 1.2	1.3 ± 0.4	82.1
M99015	57.5 ± 16.2	35.0 ± 15.9	51.4 ± 12.1	35.4 ± 10.8	38.4
M98019	5.1 ± 0.9	3.1 ± 1.3	3.2 ± 0.8	3.6 ± 1.1	29.5
M98025	15.1 ± 2.4	5.1 ± 0.6	5.0 ± 1.8	3.7 ± 3.4	75.5
M99021	13.7 ± 3.2	14.3 ± 1.5	15.3 ± 3.8	10.2 ± 5.4	25.2
M00012	21.0 ± 4.5	14.7 ± 1.8	16.4 ± 3.5	10.9 ± 3.3	48.3
M98026	54.5 ± 29.7	47.3 ± 26.0	42.5 ± 21.4	32.5 ± 18.2	40.3
M99004	4.8 ± 0.5	2.8 ± 0.6	2.3 ± 1.0	1.9 ± 0.9	60.0
M99016	34.0 ± 3.4	23.9 ± 3.6	25.5 ± 2.6	12.5 ± 1.4	63.2
M99006	17.3 ± 3.7	13.6 ± 3.3	15.5 ± 5.8	12.6 ± 4.1	27.5
M00004	33.4 ± 6.1	27.1 ± 13.7	28.3 ± 9.6	19.8 ± 12.3	40.7
M98035	5.9 ± 3.9	2.7 ± 1.1	5.7 ± 1.6	2.7 ± 1.3	54.7
M98030	9.5 ± 1.5	8.1 ± 3.3	5.5 ± 1.8	4.0 ± 1.6	57.7
M99005	2.5 ± 1.3	1.3 ± 1.2	2.2 ± 2.3	1.5 ± 1.7	38.8
M99025	3.2 ± 0.6	3.2 ± 0.2	2.5 ± 1.3	1.4 ± 0.1	57.1
M98016	53.4 ± 2.5	26.1 ± 2.3	25.6 ± 7.0	7.0 ± 1.4	86.8
M98013	23.1 ± 2.0	14.5 ± 2.8	12.2 ± 1.2	3.0 ± 0.9	87.0

^a T = Treatment groups as described in Section 2.
^b Number of adducts per 10⁸ nucleotides.
^c Percentage (%) reduction in number of adducts in the T4 group compared to the T1 group.

breast., It exhibits weak induction by aryl-hydrocarbon receptor (AhR) ligands. This may explain the relatively lower induction of *CYP1B1* compared to *CYP1A1* in our study, and may contribute to the inter-individual variability in inducible expression levels observed [18].

In this study, chlorophyllin suppressed the BP-mediated induction of both *CYP1A1* and *CYP1B1* expression in almost all 20 of the NHMEC strains. Inhibition was observed in the group of cells co-treated with BP and chlorophyllin (group T2), as well as when cells were pre-treated with chlorophyllin alone, washed and subsequently treated with BP (group T3). Other *in vivo* studies also found highly effective inhibition when chlorophyllin was co-administered with the carcinogen [19]. These data suggest that chlorophyllin might be acting by at least two different mechanisms. In previous studies, chlorophyllin has been found to form

tight molecular complexes with planar aromatic hydrocarbons, possessing at least partial ring structure, when a large molar excess of chlorophyllin was added simultaneously with the carcinogen [20]. In addition, complex formation between BPDE and chlorophyllin facilitates BP tetrol foramtion limiting the amount of BPDE [21], contributing to the reduction of BPdG adducts as well as BP-induced *CYP1A1* and *CYP1B1* expression when chlorophyllin was given simultaneously with BP. A reduction in expression in most NHMEC strains upon treatment with chlorophyllin prior to carcinogen exposure (treatment group T3) suggests mitigation of cytochrome P450 expression by mechanisms other than just carcinogen sequestration. Chlorophyllin-induced modulation of *CYP1* expression via mechanisms independent of “carcinogen sequestration” might occur through inhibition or reduction of the basal levels of NADPH-cytochrome c reductase [21], or modulation of the levels of Phase I enzymes directly or indirectly by altering expression of genes involved in the AHR pathway. These mechanisms could occur as a result of chlorophyllin exposure and continue to be active even after chlorophyllin is removed from the medium and BP is added (as in group T3). Therefore, the reduction of BP-induced *CYP1* expression in NHMEC treatment group T4 is consistent with the role of chlorophyllin as an interceptor molecule as well as a possible indirect modulator of *CYP1* enzymes.

In conclusion, the lack of correlation between the suppression of *CYP1A1* and *CYP1B1* induction and reduction in DNA adduct levels suggests that the modulation of human DNA adduct levels may be partially mediated independently of cytochrome P450 induction. Additionally, the extent of adduct formation and sensitivity of detection of adducts may be different from that of *CYP1* gene expression thereby at least partially contributing to the discrepancy. In this study, in every single cell strain,

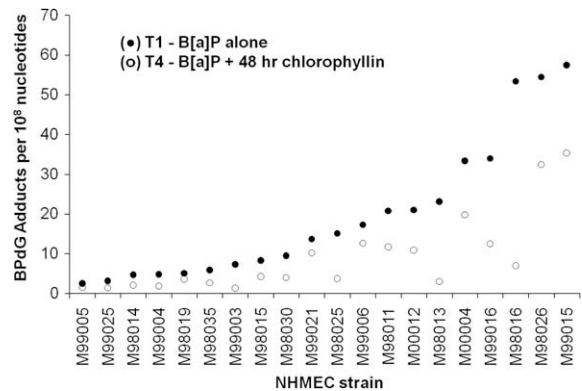


Fig. 3. Pairwise BPdG adduct values (per 10⁸ nucleotides) for 20 NHMEC strains exposed to either BP alone (T1 group, ●) and or BP plus chlorophyllin (T4 group, ○). Adduct values for each strain are ranked in order of extent of BPdG formation in the T1 group.

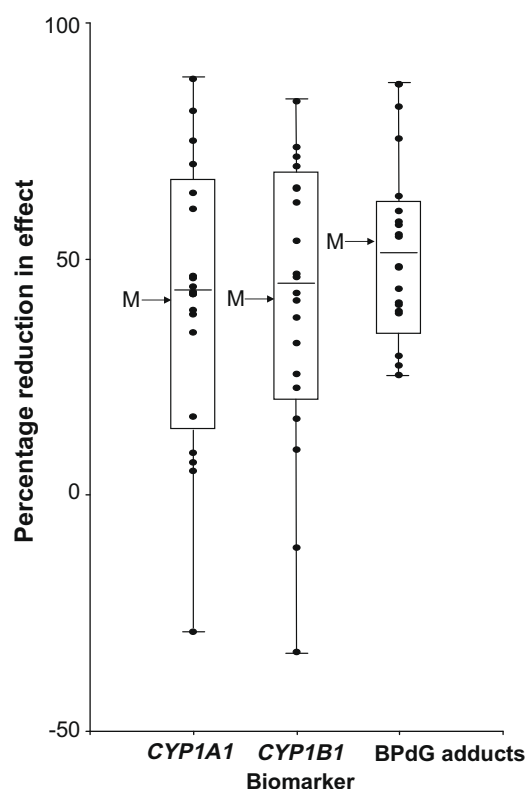


Fig. 4. Percentage reduction in *CYP1A1* and *CYP1B1* expression levels and benzo[a]pyrene-diol-epoxide-DNA adduct (BPdG) levels in the presence of chlorophyllin for normal human mammary epithelial cells from 20 individual donors that were exposed to benzo[a]pyrene in vitro. This box and whisker plot indicates the median percentage and 25% and 75% percentage changes, and outliers are indicated by the whiskers. The mean values are indicated by arrows marked M, these values are 41.1 (± 6.5 SE) for *CYP1A1*, 41.0 (± 6.7 SE) for *CYP1B1*, and 53 (± 4.2 SE) for BPdG. These changes were statistically significant $p < 0.005$ for *CYP1A1*, $p < 0.005$ for *CYP1B1* and $p < 0.0005$ for BPdG adducts.

chlorophyllin protected against BPdG adduct formation to some degree (25–87%). Therefore, chlorophyllin is likely to be an effective chemoprotective agent for a large proportion of the human population.

Conflict of interest

None of the authors have any financial or other conflict of interest.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Cancer Institute or the National Institute for Occupational Safety and Health.

Acknowledgments

Thanks to Kathy Boyce and Melanie Moore for clerical assistance. We also gratefully acknowledge the Cooperative Human Tissue Network (sponsored by the National

Cancer Institute and the National Disease Research Interchange) for providing normal human mammary tissues with which we developed the NHMEC strains. This work was supported by the intramural program of the Center for Cancer Research, National Cancer Institute, NIH (Bethesda, MD), West Virginia University, and the intramural program of the National Institute for Occupational Safety and Health, CDC (Morgantown, WV).

References

- [1] H.C. Brandt, W.P. Watson, Monitoring human occupational and environmental exposures to polycyclic aromatic compounds, *Ann. Occup. Hyg.* 47 (2003) 349–378.
- [2] T. Shimada, Y. Fujii-Kuriyama, Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1, *Cancer Sci.* 95 (2004) 1–6.
- [3] P. Sims, P.L. Grover, A. Swaisland, K. Pal, A. Hewer, Metabolic activation of benzo[a]pyrene proceeds by a diol-epoxide, *Nature* 252 (1974) 326–328.
- [4] D. Sarkar, A. Sharma, G. Talukder, Chlorophyll and chlorophyllin as modifiers of genotoxic effects, *Mutat. Res.* 318 (1994) 239–247.
- [5] P.A. Egner, J.B. Wang, Y.R. Zhu, B.C. Zhang, Y. Wu, Q.N. Zhang, G.S. Qian, S.Y. Kuang, S.J. Gange, L.P. Jacobson, K.J. Helzlsouer, G.S. Bailey, J.D. Groopman, T.W. Kensler, Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer, *Proc. Natl. Acad. Sci. USA* 98 (2001) 14601–14606.
- [6] T.W. Kensler, P.A. Egner, J.B. Wang, Y.R. Zhu, B.C. Zhang, P.X. Lu, J.G. Chen, G.S. Qian, S.Y. Kuang, P.E. Jackson, S.J. Gange, L.P. Jacobson, A. Munoz, J.D. Groopman, Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas, *Gastroenterology* 127 (2004) S310–S318.
- [7] J.W. Fahey, K.K. Stephenson, A.T. Dinkova-Kostova, P.A. Egner, T.W. Kensler, P. Talalay, Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes, *Carcinogenesis* 26 (2005) 1247–1255.
- [8] S.S. Kumar, B. Shankar, K.B. Sainis, Effect of chlorophyllin against oxidative stress in splenic lymphocytes in vitro and in vivo, *Biochim. Biophys. Acta* 1672 (2004) 100–111.
- [9] J.E. Mata, Z. Yu, J.E. Gray, D.E. Williams, R. Rodriguez-Proteau, Effects of chlorophyllin on transport of dibenzo[a,l]pyrene, 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine, and aflatoxin B(1) across Caco-2 cell monolayers, *Toxicology* 196 (2004) 117–125.
- [10] P.L. Grover, A.D. MacNicoll, P. Sims, G.C. Easty, A.M. Neville, Polycyclic hydrocarbon activation and metabolism in epithelial cell aggregates prepared from human mammary tissue, *Int. J. Cancer* 26 (1980) 467–475.
- [11] C. Keshava, D. Whipkey, A. Weston, Transcriptional signatures of environmentally relevant exposures in normal human mammary epithelial cells: benzo[a]pyrene, *Cancer Lett.* 221 (2005) 201–211.
- [12] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, *Nat. Protoc.* 3 (2008) 1101–1108.
- [13] P.W. Laird, A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, A. Berns, Simplified mammalian DNA isolation procedure, *Nucleic Acids Res.* 19 (1991) 4293.
- [14] R.L. Divi, F.A. Beland, P.P. Fu, L.S. Von Tungeln, B. Schoket, J.E. Camara, M. Ghei, N. Rothman, R. Sinha, M.C. Poirier, Highly sensitive chemiluminescence immunoassay for benzo[a]pyrene–DNA adducts: validation by comparison with other methods, and use in human biomonitoring, *Carcinogenesis* 23 (2002) 2043–2049.
- [15] D.B. Saunders, R.G. Trapp, Association and prediction, in: J. Dolan, C. Lagan (Eds.), *Basic and Clinical Biostatistics*, second ed., Appleton and Lange, Norwalk, CT, 1994, pp. 162–187.
- [16] C. Keshava, R.L. Divi, T.L. Einem, D.L. Richardson, S.L. Leonard, N. Keshava, M.C. Poirier, A. Weston, Chlorophyllin significantly reduces benzo[a]pyrene–DNA adduct formation and alters cytochrome P450 1A1 and 1B1 expression and EROD activity in normal human mammary epithelial cells, *Environ. Mol. Mutagen.* 50 (2009) 134–144.
- [17] R. Goth-Goldstein, M.R. Stampfer, C.A. Erdmann, M. Russell, Interindividual variation in CYP1A1 expression in breast tissue and the role of genetic polymorphism, *Carcinogenesis* 21 (2000) 2119–2122.
- [18] Q. Ma, A.Y. Lu, Origins of individual variability in P4501A induction, *Chem. Res. Toxicol.* 16 (2003) 249–260.

- [19] R.H. Dashwood, The importance of using pure chemicals in (anti) mutagenicity studies: chlorophyllin as a case in point, *Mutat. Res.* 381 (1997) 283–286.
- [20] R. Dashwood, Chlorophylls as anticarcinogens, *Int. J. Oncol.* 10 (1997) 721–727.
- [21] N. Tachino, D. Guo, W.M. Dashwood, S. Yamane, R. Larsen, R. Dashwood, Mechanisms of the in vitro antimutagenic action of chlorophyllin against benzo[a]pyrene: studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen, *Mutat. Res.* 308 (1994) 191–203.