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Benzo[a]pyrene (BP) DNA adduct formation in DNA repair–deficient p53 haploinsufficient [*Xpa(−/−)p53(+/−)*] and wild-type mice fed BP and BP plus chlorophyllin for 28 days

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We have evaluated DNA damage (DNA adduct formation) after feeding benzo[*a*]pyrene (BP) to wild-type (WT) and cancer-susceptible *Xpa*(−/−)*p53*(+/−) mice deficient in nucleotide excision repair and haploinsufficient for the tumor suppressor *p53*. DNA damage was evaluated by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ES-MS/MS), which measures r7,t8,t9-trihydroxy-c-10-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG), and a chemiluminescence immunoassay (CIA), using anti-r7,t8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE)–DNA antiserum, which measures both BPdG and the other stable BP-DNA adducts. When mice were fed 100 ppm BP for 28 days, BP-induced DNA damage measured in esophagus, liver and lung was typically higher in *Xpa*(−/−)*p53*(+/−) mice, compared with WT mice. This result is consistent with the previously observed tumor susceptibility of *Xpa*(−/−)*p53*(+/−) mice. BPdG, the major DNA adduct associated with tumorigenicity, was the primary DNA adduct formed in esophagus (a target tissue in the mouse), whereas total BP-DNA adducts predominated in higher levels in the liver (a non-target tissue in the mouse). In an attempt to lower BP-induced DNA damage, we fed the WT and *Xpa*(−/−)*p53*(+/−) mice 0.3% chlorophyllin (CHL) in the BP-containing diet for 28 days. The addition of CHL resulted in an increase of BP–DNA adducts in esophagus, liver and lung of WT mice, a lowering of BPdG in esophagi of WT mice and livers of *Xpa*(−/−)*p53*(+/−) mice and an increase of BPdG in livers of WT mice. Therefore, the addition of CHL to a BP-containing diet showed a lack of consistent chemoprotective effect, indicating that oral CHL administration may not reduce PAH–DNA adduct levels consistently in human organs.

Abbreviations: ANOVA, analysis of variance; BP, benzo[a]pyrene; BPDE, r7,t8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BPDE–DNA CIA, chemiluminescence immunoassay utilizing antiserum elicited against DNA modified with BPDE; BPdG, r7,t8,t9-trihydroxy-c-10-(N2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[a]pyrene; BP–DNA, DNA modified with stable adducts of BP; CHL, chlorophyllin; CYP, cytochrome P450; HPLC/ES-MS/MS, high-performance liquid chromatography/electrospray ionization tandem mass spectrometry; LOD, lower limit of detection; NER, nucleotide excision repair; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; *p53(+/−)*, mice heterozygous for the p53 gene; *Xpa (−/−)*, mice null for the Xpa gene; XP, Xeroderma pigmentosum; WT, wild type.

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Introduction

Benzo[*a*]pyrene (BP), a polycyclic aromatic hydrocarbon (PAH), is a widely distributed environmental carcinogen. Being a ubiquitous pollutant, exposure to BP can occur through diet, occupation and/or lifestyle. Following exposure, BP undergoes metabolism by Phase I enzymes, predominantly cytochrome P450s (CYPs), to various genotoxic metabolites (1). These metabolites can either react with cellular macromolecules or be converted to more hydrophilic intermediates that are subsequently excreted (2). A major BP metabolite, r7,t8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), binds deoxyguanosine in DNA to form, primarily, r7,t8,t9-trihydroxy-c-10-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG) (3). Other stable adducts (4) and a number of unstable adducts (5) have also been reported. The formation of BPdG adducts in various key tumor suppressor genes, oncogenes and gatekeeper genes, leading to mutations, has been considered to be important in initiating the multi-step process of chemical carcinogenesis (6). However, because DNA adduct formation is considered necessary but not sufficient for tumor formation, the precise relationship between exposure, genetic insult and disease is not always straightforward (6).

Biological organisms utilize various protective mechanisms in response to carcinogen exposure, including metabolism to water-soluble derivatives by 'Phase II' enzyme reactions, elimination of highly damaged cells via apoptosis and the removal of DNA damage via complex DNA repair mechanisms (2,7,8). Nucleotide excision repair (NER) likely evolved for removal of DNA damaging UV-induced photo lesions, but also efficiently removes large molecular weight helix-distorting DNA adducts such as the BPdG (9). The inability to repair UV-induced lesions because of defects in the NER pathway results in the disease *Xeroderma pigmentosum* (XP), an inherited autosomal recessive disorder characterized by excessive sensitivity of skin to UV radiation and accelerated skin cancer induction. There are 30–40 different polypeptides involved in the process of NER, and the XP complementation group A (Xpa) protein is thought to have a key role in binding to damaged DNA and positioning the repair machinery around the lesion (10,11). *Xpa*-deficient mice lack almost all NER and are susceptible to enhanced cancer induction when exposed to various chemical carcinogens, including BP (10,12–15). p53, a multi-functional tumor suppressor protein, is mutated in many cancers (16). In part, p53 protects against tumor induction by delaying the replication of damaged DNA to allow for damage removal by NER (17). Therefore, transgenic mice deficient in both *Xpa* and *p53* constitute an attractive model for the study of genotoxic carcinogens such as BP. Compared with their wild-type (WT) counterparts, *Xpa*(−/−)*p53*(+/−) mice exhibit enhanced susceptibility to chemical carcinogen-induced tumors (16,18–21).

Given that there is a need to understand mechanisms underlying human cancer induction by environmental pollutants, we examined DNA adduct processing in esophagi, livers and lungs of WT and *Xpa*(−/−)*p53*(+/−) mice fed BP for 28 days, a time period known to produce steady-state DNA adduct formation (22–24). In addition, because of our interest in chemoprevention, we assessed BP–DNA adduct formation in the presence of chlorophyllin (CHL), a water soluble sodium–copper salt of chlorophyll. Based on literature reports in rodents, fish and cell culture, we hypothesized that CHL would reduce BP–DNA adduct formation in target organs, thus protecting them against tumorigenesis (25–31). We used two methods to measure DNA damage induced by BP: high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ES-MS/MS), which determined only the major adduct, BPdG (32); and anti-BPDE–DNA chemiluminescence immunoassay (CIA),

a competitive ELISA using an antiserum specific for BPdG and the other BP–DNA adducts. For both WT and *Xpa*(−/−)*p53*(+/−) mice, we examined unexposed groups as well as groups exposed to BP alone, BP plus CHL and CHL alone. The resulting DNA adducts were assessed in esophagus, liver and lung.

Materials and methods

Mouse derivation, breeding and genotyping

Xpa(−/−)*p53*(+/−) mice, kindly provided by H. van Steeg of the Laboratory of Toxicology, Bilthoven, Netherlands, were generated on a C57BL/6 background as described previously (33–35). Control C57BL/6 mice, WT for both *Xpa* and *p53*, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mouse offspring were paw tattooed and tail clipped 8–10 days following birth. DNA from the tail clips was used to genotype the mice, using primer sequences for *Xpa* and *p53* that have been published (15,35).

Mice were housed in a pathogen-free, climate-controlled facility with a 12 h light/dark cycle. Dietary exposure was started at 6–8 weeks of age and carried out for a total of 4 weeks. Animals were subject to one of the following treatment regimes: powdered NIH 31 diet (control); powdered NIH 31 diet containing 100 ppm BP; powdered NIH 31 diet containing 100 ppm BP + 0.3% (3000 ppm) CHL or powdered NIH 31 diet containing 0.3% CHL alone. All animals were provided food and water *ad libitum*. Per treatment group, 4–10 animals were used with approximately equal numbers of males and females. Protocols were approved by the National Cancer Institute Animal Care and Use Committee, and mice were maintained under AAALAC-approved conditions.

DNA extraction

Following 4 weeks of dietary exposure, mice were euthanized and tissues flash frozen and stored until DNA was extracted. Upon thawing, tissues were homogenized (Ultra Turrax T10, Ika Works, Wilmington, NC) in ice-cold PBS (pH 7.4) and incubated overnight at 37°C with an equal volume of buffer ATL (Qiagen, Valencia, CA) containing RNase A and proteinase K. The following day, DNA was extracted from the esophagi, lungs and livers using DNeasy Blood Maxi kits (Qiagen), according to the manufacturer's instructions.

DNA adduct analyses

Two approaches were used to evaluate the levels of DNA damage. For the first approach, the BPdG adduct was analyzed by HPLC/ES-MS/MS, as described previously (32). The HPLC/ES-MS/MS method, as used here, assessed only the BPdG adduct.

The second approach involved a CIA using a rabbit polyclonal antiserum elicited against BPDE-modified DNA as described (36,37). For this assay, although the immunogen was DNA modified with BPdG, the antiserum cross-reacts with a family of carcinogenic PAHs bound to DNA (38), as well as all the stable DNA adducts of BP.

The BPDE–DNA CIA was performed in an opaque 96-well high-binding plates (Greiner Bio-one, Longwood, FL) coated with 100 pg of sonicated BPDE–DNA (modified to 0.33%), or calf thymus DNA, in 100 µl of Reacti-Bind DNA coating solution (Pierce, Thermo Fisher Scientific, Pittsburgh, PA) (36). For BPDE–DNA CIA analysis, plates were washed with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBST) and 0.02% sodium azide, using an automated plate washer (Ultrawash Plus, Dynex, Chantilly, VA). Plates were blocked with casein (0.25%; Applied Biosystems, Bedford, MA) in PBST at 37°C for 90 min to reduce non-specific binding. Sample DNA (0.1–2.5 µg/well for WT mice; 0.1–1.75 µg/well for *Xpa*(−/−)*p53*(+/−) mice) and standard BPDE–DNA were sonicated (30 seconds at 20% amplitude using an Ultrasonic Processor, Sonics & Materials, Inc., Newtown, CT) and mixed with an equal volume of PAH–DNA antiserum (rabbit # 31, bleed 08/16/78) diluted 1:3,000,000 in PBST-containing casein (0.25%). Standard BPDE–DNA, modified to 1.0 BPdG/10^6 nucleotides, was serially diluted in calf-thymus DNA so that each well contained an equal quantity of DNA but varying amounts of BPdG adduct (0–8.0 fmol/well). After adding samples and standards to wells, plates were incubated at 37°C for 90 min followed by washing with PBST and incubation with biotinylated anti-rabbit antibody (1:2500; Jackson ImmunoResearch Laboratories, West Grove, PA) in PBST-containing casein (0.25%) at room temperature for 60 min. After washing, the plates were incubated with streptavidin alkaline phosphatase (1:5000; Avidix–AP, Applied Biosystems) in PBST-containing casein (0.25%) at room temperature for 60 min. The plates were washed sequentially with PBST, distilled water and Tris buffer (20 mM Tris and 1 mM $MgCl_2$, pH 9.5) before adding CDP Star with Emerald II solution (Applied Biosystems, Carlsbad, CA). After an overnight incubation at 37°C, the plates were brought to room temperature and luminescence was read using a Tropix 717 Microplate Luminometer (Applied Biosystems). For each assay, sample DNA was incubated in three experimental wells and one control well. The Standard Curve 50% Inhibition was at 0.343 ± 0.046 fmol (mean ± SE, n = 18). The lower limit of detection (LOD) varied, based on the tissue and the amount of DNA available for assay. The LOD range was 1.5–9.8 adducts/10^8 nucleotides for esophagus, 1.0–2.3 adducts/10^8 nucleotides for lung and 2.3–10.8 adducts/10^8 nucleotides for liver.

Statistical analyses

Statistical comparisons of DNA adduct levels between genotypes, treatments or sexes were conducted by a one-way analysis of variance (ANOVA), with pairwise comparisons performed by the Student–Newman–Keuls method. When necessary, the DNA adduct data were natural log transformed before the analyses to maintain a more equal variance or normal data distribution. Statistical analyses of DNA adduct levels among tissues were conducted by one-way repeated measures ANOVA and the Student–Newman–Keuls method.

Results

DNA adduct formation in WT and Xpa(−/−)p53(+/−) mice fed BP: Esophagus

Figure 1A shows values for BPdG, as determined by HPLC/ES-MS/MS, and total BP-DNA adducts, as determined by BPDE–DNA CIA, in the esophagus of WT and *Xpa*(−/−)*p53*(+/−) mice. Whereas the figure shows males (○) and females (●) separately, when both males and females were considered together, the data indicated that the *Xpa*(−/−)*p53*(+/−) mice had increased levels of BP-induced DNA damage, compared with the WT mice, regardless of DNA adduct analysis method. Also, despite a 2-fold lower value being obtained in WT mice with BPDE–DNA CIA, compared with HPLC/ES-MS/MS ($P < 0.05$), the data suggest that the major/only DNA adduct formed in esophagus is the BPdG.

DNA adduct formation in WT and Xpa(−/−)p53(+/−) mice fed BP: Liver

Figure 1B shows values for BPdG, as determined by HPLC/ES-MS/MS, and total BP–DNA adducts, as determined by BPDE–DNA CIA, in the livers of WT and *Xpa*(−/−)*p53*(+/−) mice. When both males and females were considered together, the data show that the *Xpa*(−/−)*p53*(+/−) mice had 5.5-fold higher levels of BPdG, compared with the WT mice. In contrast, total BP–DNA adducts, as measured by BPDE–DNA CIA, did not show a statistically significant difference with genotype. Furthermore, as opposed to the esophagus, where both DNA adduct determination methods likely detected primarily BPdG, in the liver the adduct levels were 21-fold higher in WT mice and 7-fold higher in *Xpa*(−/−)*p53*(+/−) mice, when measured by BPDE–DNA CIA as compared with HPLC/ES-MS/MS.

DNA adduct formation in WT and Xpa(−/−)p53(+/−) mice fed BP: Lung

Figure 1C shows values for BPdG, as determined by HPLC/ES-MS/MS, and total BP–DNA adducts, as determined by BPDE–DNA CIA, in the lungs of WT and *Xpa*(−/−)*p53*(+/−) mice. When both males and females were considered together, the data for lungs show that *Xpa*(−/−)*p53*(+/−) mice have 2-fold higher levels of total BP-induced DNA damage, as determined by BPDE–DNA CIA, compared with the WT mice; however, the levels of BPdG adducts, as determined by HPLC/ES-MS/MS, were nearly identical in mice of both genotypes. In the lungs, total BP–DNA adducts, as assessed by BPDE–DNA CIA, were 2- and 3-fold higher than the BPdG levels determined by HPLC/ES-MS/MS, in WT and *Xpa*(−/−)*p53*(+/−) mice, respectively. Therefore, approximately 30–56% of the total lung BP–DNA adducts may consist of BPdG. These data suggest that the lung forms stable BP–DNA adducts in addition to BPdG, but not to the same extent as was observed in the liver.

Comparison of DNA adduct levels in the different organs of BP-exposed mice

When assessed by HPLC/ES-MS/MS, with males and females combined, BPdG levels decreased in the order esophagus > lung > liver in WT mice fed BP, and in the order esophagus > liver ≈ lung in

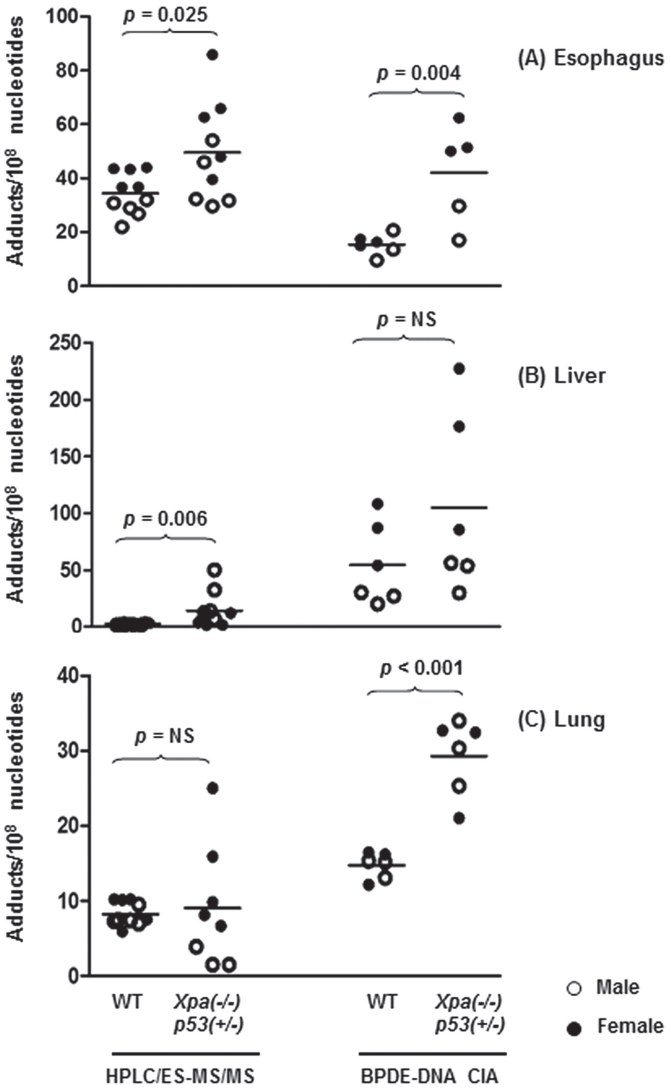

Fig. 1. DNA adducts (per 10^8 nucleotides) induced in (**A**) esophagus, (**B**) liver and (**C**) lung of WT and *Xpa(−/−)p53(+/−)* male (○) and female (●) mice fed 100 ppm BP in the diet for 28 days. HPLC/ES-MS/MS was used to measure BPdG, and BPDE–DNA CIA was used to measure total BP–DNA adducts. There were no DNA adducts found in the unexposed controls (data not shown). The p-values shown are for statistical comparisons between WT and *Xpa(−/−)p53(+/−)* mice. NS = not significant.

Xpa(−/−)*p53*(+/−) mice fed BP (Table I). By BPDE–DNA CIA, the levels for total BP–DNA adducts in mice fed BP showed a different pattern (Table II), with a ranking of liver > esophagus ≈ lung for WT mice and liver > esophagus > lung for *Xpa*(−/−)*p53*(+/−) mice.

Effect of CHL on DNA adduct formation in WT and Xpa(−/−) p53(+/−) mice fed BP or BP + CHL

In planning the CHL studies, we hypothesized that the level of DNA damage in target organs would decrease in mice fed BP and CHL. Table I shows a comparison, for males and females combined, for BPdG adducts determined by HPLC/ES-MS/MS in WT and *Xpa*(−/−)*p53*(+/−) mice fed BP or BP + CHL for 28 days. Table II shows a comparison, for males and females combined, for BP–DNA adducts determined by BPDE–DNA CIA in WT and *Xpa*(−/−)*p53*(+/−) mice fed BP or BP + CHL for 28 days. In WT mice, the addition of CHL to the diet caused statistically significant increases in total BP–DNA adduct levels in all three tissues (Table II),

Table I. Comparison, for males and females combined, of BPdG (per 10^8 nucleotides, mean ± SE), measured by HPLC/ES-MS/MS, in WT and *Xpa*(−/−)*p53*(+/−) mice fed BP or BP plus CHL

Mice	Organ	BP diet[a]	BP + CHL diet[a]	*P* values
WT	Esophagus	34.4 ± 2.4 (10)	23.8 ± 3.3 (6)	**0.020**[b]
WT	Liver	2.6 ± 0.4 (10)	8.7 ± 0.8 (6)	**<0.001**[c]
WT	Lung	8.3 ± 0.5 (10)	10.2 ± 1.2 (6)	NS[bc]
Xpa(−/−)*p53*(+/−)	Esophagus	49.6 ± 5.7 (10)	42.9 ± 2.7 (5)	NS
Xpa(−/−)*p53*(+/−)	Liver	14.3 ± 4.9 (10)	2.4 ± 1.1 (5)	**0.019**
Xpa(−/−)*p53*(+/−)	Lung	9.1 ± 2.8 (8)	6.0 ± 1.1 (5)	NS

[a]Numbers of mice are shown in parentheses.
[b] Statistical significance is indicated in bold
[c]NS = not significant ($P > 0.05$) for BP versus BP + CHL comparison.

Table II. Comparison, for males and females combined, of BP-DNA adducts (per 10^8 nucleotides, mean ± SE), measured by BPDE-DNA CIA, in WT and *Xpa*(−/−)*p53*(+/−) mice fed BP or BP plus CHL

Mice	Organ	BP diet[a]	BP + CHL diet[a]	*P* values
WT	Esophagus	15.4 ± 1.5 (6)	65.1 ± 8.7 (6)	**<0.001**[b]
WT	Liver	54.5 ± 14.7 (6)	277.5 ± 31.7 (5)	**<0.001**
WT	Lung	14.7 ± 0.7 (6)	19.1 ± 1.2 (6)	**0.012**
Xpa(−/−)*p53*(+/−)	Esophagus	42.1 ± 8.2 (5)	36.7 ± 3.7 (5)	NS[c]
Xpa(−/−)*p53*(+/−)	Liver	104.9 ± 32.2 (6)	36.7 ± 13.7 (4)	NS
Xpa(−/−)*p53*(+/−)	Lung	29.2 ± 2.1 (6)	35.1 ± 7.8 (5)	NS

[a]Numbers of mice are shown in parentheses.
[b] Statistical significance is indicated in bold
[c]NS = not significant ($P > 0.05$) for BP versus BP + CHL comparison.

with a 5.2-fold increase in liver, a 4.1-fold increase in esophagus and a 1.3-fold increase in lung. In *Xpa*(−/−)*p53*(+/−) mice, there were no significant alterations in total BP–DNA adduct levels in any of the organs of mice fed BP + CHL in the diet, compared with BP alone.

There were no adducts detected, by either HPLC/ES–MS/MS or BPDE–DNA CIA, in mice of either genotype fed CHL alone for 28 days.

Sex differences in DNA adduct formation

By HPLC/ES–MS/MS, for the mice fed BP alone, WT females had higher BPdG levels in esophagus and liver than WT males ($P < 0.05$) (Table III). In *Xpa*(−/−)*p53*(+/−) mice, females had higher levels of BPdG in esophagus than the males ($P < 0.05$) (Table III). In mice of both genotypes fed BP + CHL there were no significant sex differences for BPdG formation.

By BPDE–DNA CIA, for livers of WT mice and esophagi of *Xpa*(−/−)*p53*(+/−) mice fed BP alone, there were significantly higher levels of total BP–DNA adducts in female mice, compared with male mice (Table IV). For mice fed BP + CHL, the only significant sex difference was an elevated level of BP–DNA adducts in lungs of male *Xpa*(−/−)*p53*(+/−) mice, compared with females.

Discussion

As expected, the *Xpa*(−/−)*p53*(+/−) mice, which are deficient in NER and partially insufficient in *p53* tumor suppressor protection, typically showed higher levels of DNA damage in esophagus, liver and lung, than their WT counterparts, when fed 100 ppm BP in the diet for 28 days. These results are consistent with data observed in several studies that administered PAHs to *Xpa*(−/−) or *Xpa*(−/−)*p53*(+/−) mice, in which the end points were DNA damage, mutagenesis or tumorigenesis (12,20,21,34). Our study was modeled on two tumor studies: one in WT mice and one in *Xpa*(−/−)*p53*(+/−) mice. Using WT mice fed 100 ppm BP for 2 years, Culp et al. showed a 98% incidence of forestomach tumors, a 59% incidence of esophageal tumors and no significant increase in lung or liver tumors (39). In addition, using the same *Xpa*(−/−)*p53*(+/−) that we employed here, Hoogervorst et al. fed 75 ppm BP for 13 weeks and monitored the mice for an additional

Table III. Comparison of BPdG (per 10^8 nucleotides, mean ± SE) between sexes, measured by HPLC/ES-MS/MS, in WT and *Xpa*(–/–)*p53*(+/–) mice fed BP or BP plus CHL

Mouse strain	Treatment	Organ	Males[a]	Females[a]	*P* values
WT	BP	Esophagus	28.0 ± 1.8 (5)	40.8 ± 1.7 (5)	**<0.001**[d]
WT	BP + CHL	Esophagus	20.9 ± 3.2 (3)	26.6 ± 6.1 (3)	NS[bc]
WT	BP	Liver	1.6 ± 0.08 (5)	3.7 ± 0.2 (5)	**<0.001**
WT	BP + CHL	Liver	7.5 ± 0.6 (3)	10.0 ± 1.2 (3)	NS
WT	BP	Lung	7.7 ± 0.5 (5)	8.8 ± 0.9 (5)	NS
WT	BP + CHL	Lung	8.7 ± 1.1 (3)	11.7 ± 1.8 (3)	NS
Xpa(–/–)*p53*(+/–)	BP	Esophagus	38.7 ± 4.8 (5)	60.4 ± 8.0 (5)	**0.048**
Xpa(–/–)*p53*(+/–)	BP + CHL	Esophagus	41.4 ± 4.2 (3)	45.0 ± 3.4 (2)	NS
Xpa(–/–)*p53*(+/–)	BP	Liver	23.2 ± 8.0 (5)	5.3 ± 2.0 (5)	NS
Xpa(–/–)*p53*(+/–)	BP + CHL	Liver	1.3 ± 0.2 (3)	3.9 ± 2.7 (2)	NS
Xpa(–/–)*p53*(+/–)	BP	Lung	2.3 ± 0.8 (3)	13.1 ± 3.4 (5)	NS
Xpa(–/–)*p53*(+/–)	BP + CHL	Lung	5.7 ± 1.3 (3)	6.3 ± 2.8 (2)	NS

[a]Numbers of mice are shown in parentheses.
[b] Statistical significance is indicated in bold
[c]NS = not significant ($P > 0.05$) for male versus female comparison.

Table IV. Comparison of total BP-DNA (per 10^8 nucleotides, mean ± SE) between sexes, measured by BPDE-DNA CIA, in WT and *Xpa*(–/–)*p53*(+/–) mice fed BP or BP plus CHL

Mouse strain	Treatment	Organ	Males[a]	Females[a]	*P* values
WT	BP	Esophagus	14.6 ± 3.3 (3)	16.2 ± 0.7 (3)	NS[b]
WT	BP + CHL	Esophagus	49.2 ± 4.2 (3)	81.0 ± 10.2 (3)	**0.045**[c]
WT	BP	Liver	25.8 ± 3.0 (3)	83.2 ± 15.9 (3)	**0.028**
WT	BP + CHL	Liver	232.3 ± 18.0 (2)	307.7 ± 45.8 (3)	NS
WT	BP	Lung	14.5 ± 0.7 (3)	15.0 ± 1.4 (3)	NS
WT	BP + CHL	Lung	17.3 ± 1.9 (3)	20.9 ± 0.8 (3)	NS
Xpa(–/–)*p53*(+/–)	BP	Esophagus	23.3 ± 6.4 (2)	54.6 ± 3.9 (3)	**0.021**
Xpa(–/–)*p53*(+/–)	BP + CHL	Esophagus	35.1 ± 6.5 (3)	39.0 ± 1.9 (2)	NS
Xpa(–/–)*p53*(+/–)	BP	Liver	46.5 ± 8.3 (3)	163.2 ± 41.5 (3)	NS
Xpa(–/–)*p53*(+/–)	BP + CHL	Liver	45.2 ± 29.3 (2)	28.3 ± 11.6 (2)	NS
Xpa(–/–)*p53*(+/–)	BP	Lung	29.9 ± 2.5 (3)	28.7 ± 3.8 (3)	NS
Xpa(–/–)*p53*(+/–)	BP + CHL	Lung	47.3 ± 4.4 (3)	16.9 ± 0.04 (2)	**0.004**

[a]Numbers of mice are shown in parentheses.
[b]NS = not significant ($P > 0.05$) for male versus female comparison.
[c]Statistical significance is indicated in bold.

6 months (34). They observed an 80% incidence of forestomach tumors and a 20% incidence of esophageal tumors, but no lung or liver tumors. Whereas the Culp study was performed using female B6C3F1 mice and the Hoogervorst study employed *Xpa*(–/–)*p53*(+/–) mice on a C57BL/6 background, the total BP dose in the WT mice fed 100 ppm BP for 2 years was substantially greater than the total BP dose in the *Xpa*(–/–)*p53*(+/–) mice fed 75 ppm BP for 13 weeks (34,39). Taken together, our DNA adduct data, and the tumor studies, support the notion that the inability to remove DNA damage induced by BP and the loss of ability to arrest DNA replication of DNA damaged with BP both contribute to the increased sensitivity for BP-induced cancers in *Xpa*(–/–)*p53*(+/–) mice, compared with WT mice, fed BP.

A second observation from these studies is related to the different DNA adducts detected by the HPLC/ES–MS/MS and BPDE–DNA CIA methods in the organs of mice fed BP for a month. Mass spectrometry-based methods are generally considered the 'gold standard' of DNA adduct measurements as they are the only approaches that can measure the chemically specific BPdG. In contrast, the immunoassays target a broader variety of structures, based on antiserum cross-reactivity. In employing both methodologies in these experiments, and comparing the results, we demonstrated that the BP-induced DNA damage formed in esophagus of BP-fed mice is primarily BPdG. In contrast, it appears that the large majority, possibly as much as 86–95%, of the adducts formed in liver and detected by BPDE–DNA CIA are not BPdG adducts, but rather multiple types of other stable BP–DNA adducts, which may be formed because of the complex metabolic capacity of the liver. Similarly, it is possible that 44–69% of the BP–DNA adducts formed in lung are not BPdG. Overall, the spectra of DNA adducts formed in liver and lung are significantly more complex than the BPdG that predominates in the esophagus. The importance of BPdG as the major stable DNA adduct associated with BP-induced tumorigenesis has been previously demonstrated in the skin, lungs and forestomach of mice (12,40,41). The role of the additional adducts in liver is unclear because BP is not normally a liver carcinogen in mice. Therefore, despite the fact that the liver formed the highest levels of stable DNA adducts in both WT and *Xpa*(–/–)*p53*(+/–) mice, it appears likely that most of the liver DNA adducts formed from BP feeding for 28 days are not associated with tumor induction.

In planning these experiments, we hypothesized that the addition of CHL to the BP-containing diet would result in lower levels of BP-induced DNA damage in mouse organs. Long-term feeding of carcinogens in experimental models has shown that DNA adduct levels are positively correlated with the tumorigenic response, indicating that lowering DNA damage levels is likely to reduce tumorigenesis (24,42,43). Previous studies in our laboratory, using 20 different strains of normal human mammary epithelial cells cultured from 20 different individuals, showed a reduction of 25–87% in BP–DNA adduct levels in cells exposed to BP + CHL, compared with cells exposed to BP alone (27). Furthermore, since humans ingesting aflatoxins in the presence of CHL showed reductions in excreted aflatoxin–DNA adducts, we hypothesized that a similar reduction in PAH–DNA adducts might be possible by co-administering CHL (44). Here, we have shown that, in mice of both genotypes, for BPdG levels determined by HPLC/ES–MS/MS, the addition of CHL to the BP-containing diet (Table I) induced significant changes, but the direction of the changes was inconsistent. BPdG levels were significantly decreased in esophagi of WT mice and livers of

Xpa(−/−)*p53*(+/−) mice fed BP + CHL, compared with mice fed BP alone; however, in livers of WT mice fed BP + CHL, there were higher levels of BPdG than in the livers of mice fed BP alone. For total BP–DNA adducts determined by BPDE–DNA CIA (Table II), the addition of CHL to BP in the diets of WT mice induced significant increases in the total BP–DNA adduct levels in all three tissues (5.1-fold in the liver, 4.2-fold in the esophagus and 1.3-fold in the lung). In *Xpa*(−/−)*p53*(+/−) mice, the addition of CHL to the BP diet produced no significant change in the total BP–DNA adduct levels. It is difficult to comprehend underlying mechanisms here, but we must conclude that the addition of CHL to the diets of mice fed BP does not result in a single consistent change and that organ-specific metabolic events may be highly relevant in determining total DNA damage formed in the presence of BP + CHL. The DNA damage levels vary by genotype and organ, suggesting that CHL may not be a consistent chemoprotective agent for mice or other animals. Possible confounding factors may include interspecies differences in metabolism and DNA damage processing, particularly as related to mice and humans. Additionally, there is the issue of carcinogenic potency of specific DNA adducts. For example, it appears that even though the livers of WT mice fed BP + CHL have very high levels of DNA damage (277.5 adducts/10^8 nucleotides, Table II), only a very small fraction of these (8.7 adducts/10^8 nucleotides, Table I) constitute BPdG, an adduct known to be associated with carcinogenesis. Since the liver is not a target organ for BP-induced tumorigenesis in the mouse, one could argue that a large fraction of the liver DNA damage induced by BP + CHL in this organ is irrelevant with respect to carcinogenesis. Nevertheless, it would be difficult to recommend CHL as a chemopreventive agent in humans if it does increase DNA damage in any organ in such a dramatic fashion.

Our findings with CHL are supported by a study in which mice were given a single oral dose of BP with or without CHL (45). These investigators found no alteration of BP–DNA adduct levels in the liver or leukocyte DNA, determined by ^{32}P-post labeling, when CHL was fed for a week prior to the administration of a single dose of BP. In addition, in mouse livers and leukocytes, pre-treatment with CHL did not reduce micronuclei, but did reduce Comet assay indicators of DNA damage. Although their use of a single BP dose was very different from our continuous feeding of BP for 4 weeks, and their dosing with CHL was only for a week, both studies conclude that CHL does not protect against the formation of BP-induced DNA damage in mice.

Overall, the studies presented here have demonstrated that the enhanced susceptibility of NER-deficient, p53-haploinsufficint mice to BP-induced tumorigenesis is at least partially due to the formation of higher levels of BPdG and total BP–DNA adducts in target organs of the transgenic mice, compared with WT mice. These studies have also indicated that the total BP–DNA adduct levels, measured by BPDE–DNA CIA, vary by organ, with liver having the highest adduct levels. However, since liver is a non-target organ for tumor induction, it appears that at least some of this DNA damage is not relevant to tumorigenesis. The addition of CHL to the BP-containing diet was expected to lower DNA damage levels, but neither the BPdG nor the total BP–DNA adduct levels was altered in a consistent fashion. Therefore, these studies do not provide evidence for a generalized chemoprotective effect of CHL in mice. On the contrary, these results suggest that human intervention with CHL should be viewed with caution, as reduction in carcinogen–DNA damage may be organ, carcinogen and/or species specific.

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