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Effect of arsenic on benzo[*a*]pyrene DNA adduct levels in mouse skin and lung

C.D.Evans[1], K.LaDow[1], B.L.Schumann[1], R.E.Savage Jr[2], J.Caruso[3], A.Vonderheide[3], P.Succop[1] and G.Talaska[1,4]

[1]University of Cincinnati, Department of Environmental Health, 3223 Eden Avenue, Cincinnati, OH 45267-0056, [2]NIRS, EPHB, DART, NIOSH, 4676 Columbia Parkway, Cincinnati, OH 45226-1998 and [3]University of Cincinnati, Department of Chemistry, 137 McMicken, Cincinnati, OH 45267-0037, USA

[4]To whom correspondence should be addressed
Email: talaskgg@ucmail.uc.edu

Concomitant exposures to arsenic and polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (BaP) are widespread. While BaP acts by binding to and inducing mutations in critical sites on DNA, the mechanism(s) of arsenic carcinogenesis remains unknown. Data from epidemiological studies of arsenic copper smelter workers and arsenic ingestion in drinking water suggest a positive interaction for arsenic exposure and smoking and lung cancer. A previous *in vitro* study showed that arsenic potentiated the formation of DNA adducts at low doses of BaP and arsenic. The present study was conducted to test the effect of arsenic on BaP–DNA adduct formation *in vivo*. We hypothesized that arsenic co-treatment would significantly increase BaP adduct levels in C57BL/6 mouse target organs: skin and lung. Treatment groups were: five mice, −BaP/−arsenic; five mice, −BaP/+arsenic; 15 mice, +BaP/−arsenic; 15 mice, +BaP/+arsenic. Mice in the appropriate groups were provided sodium arsenite in drinking water (2.1 mg/l), *ad libitum*, for 13 days (starting 9 days before BaP treatment), and 200 nmol BaP/25 ml acetone (or acetone alone) was applied topically, once per day for 4 days. DNA was extracted from skin and lung and assayed by ^{32}P-postlabeling. Statistical comparisons were made using independent *t*-tests (unequal variances assumed). BaP–DNA adduct levels in the +BaP groups were significantly higher than −BaP controls. Arsenic co-treatment increased average BaP adduct levels in both lung and skin; the increase was statistically significant in the lung ($P = 0.038$). BaP adduct levels in the skin of individual animals were positively related to skin arsenic concentrations. These results corroborate our *in vitro* findings and provide a tentative explanation for arsenic and PAH interactions in lung carcinogenesis.

Introduction

Arsenic and benzo[*a*]pyrene (BaP) are widespread contaminants in the environment. Several epidemiological studies have indicated that smoking and arsenic co-exposure may be linked to elevated rates of lung cancer, particularly in copper smelter workers. However, the literature is inconsistent; reports range from a synergistic interaction (1,2), positive interaction, but less than multiplicative effects (3) and investigations showing no effect (4–6). Additional support for a synergistic interaction between arsenic and polycyclic aromatic hydrocarbons (PAH) comes from reports of epidemiological studies of chronic arsenic exposure in drinking water and smoking (7,8). Two *in vivo* animal studies, conducted by intratracheal instillation co-exposure, also indicated that arsenic enhances BaP lung tumorigenesis. However, predicting BaP's contribution, if any, to the observed lung cancer association with smoking is problematic, as tobacco smoke is itself a complex mixture of numerous PAHs and other known carcinogens such as aromatic amines and heavy metals (9).

While BaP acts by binding to and mutating critical sites on DNA, the mechanism(s) of arsenic carcinogenesis remains unknown. Arsenic is a weak mutagen, as evidenced by the inability to form point mutations with DNA in bacteria and Chinese hamster cells (10). Several studies have reported that arsenic enhances the mutagenicity of other DNA-binding agents during the initiation stage. Arsenicals are co-mutagenic with DNA damaging agents including ultraviolet light (11,12) *N*-methyl-*N*-nitrosourea (13) and BaP (14). These *in vitro* findings suggest that arsenic exhibits significant activity during the initiation phase by modulating the genotoxicity of initiating agents. Other proposed potential mechanisms for arsenic-induced lung cancer include effects on DNA repair, DNA methylation, cytochrome P450 enzymes, glutathione homeostasis and generation of oxygen radicals (15–18).

In an *in vitro* study performed earlier, we reported that arsenic could potentiate BaP adduct formation and similarly enhance BaP mutagenesis at the *HPRT* (hypoxanthine-guanine phosphoribosyl transferase) locus in mouse Hepa-1 hepatoma cells (14). As much as a 17-fold increase in BaP adduct levels for a given BaP dose was seen when Hepa-1 cells were also exposed to low concentrations of sodium arsenite. A proportional increase in the *HPRT* forward mutation resistance to 6-thioguanine following BaP and arsenic treatment was also seen (14). While CYP450 was required for the effect, the glutathione status of the cells was very important. Interestingly, arsenic did not alter the rate of DNA repair in the cells.

This study was conducted to determine whether the results obtained *in vitro* might also be seen in the whole animal in a mouse model. These studies were done as a requisite to investigations of the mechanisms of arsenic–PAH interactions.

Materials and methods

Materials

Sodium arsenite, BaP, phosphodiesterase II, apyrase, ribonuclease A, micrococcal endonuclease/spleen phophodiesterase, adenosine 5′-triphosphate, nuclease P1, lithium hydroxide, urea, trizma® hydrochloride, bicine and sodium hydroxide solution, spermidine, dithiothreitol, magnesium chloride

Abbreviations: BaP, benzo[*a*]pyrene; ICP-MS, inductively coupled argon plasma, atomic emission coupled mass spectroscopy; PAH, polycyclic aromatic hydrocarbons; RAL, relative adduct levels.

and sodium succinate were purchased from Sigma Co. (St Louis, MO). [^{32}P]H_3PO_4 was acquired from Perkin-Elmer, NEN Life Science (Boston, MA). T4 polynucleotide kinase was obtained from United States Biochemical Corporation (Cleveland, OH). Lithium chloride, sodium phosphate monobasic, calcium chloride and 90% laboratory grade formic acid were obtained from Fisher-Scientific (Pittsburgh, PA). The Wizard Genomic kit manufactured by Promega (Madison, WI) was also purchased from Fisher Scientific. Cellulose polyethyleneimine plates were purchased from Scientific Adsorbents (Atlanta, GA). Plates were pre-washed with tap water to remove impurities. Fuji, Super RX, medical X-ray film was purchased from Philips Medical (Highland Heights, OH). Arsenic for the inductively coupled plasma (ICP) analysis calibration standards was purchased in solution from SPEX Certiprep (Metuchen, NJ).

Animal treatment

Forty female C57BL/6 mice aged 4–5 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). The dosing regimen was as follows: five mice, −BaP/−arsenic; five mice, −BaP/+arsenic; 15 mice, +BaP/−arsenic; 15 mice, +BaP/+arsenic. Upon arrival the mice were housed in clear plastic shoebox cages (five mice in the same treatment group per cage) with Bed-o' Cobs Combination Bedding (Maumee, OH) in the AAALAC-approved Kettering Laboratory animal housing facilities. Mice were allowed to acclimate 4 days prior to start of any of the treatments. Animal housing conditions were controlled for temperature (72°F), relative humidity (42%) and light/dark hours (12 h cycles, with the light cycle beginning at 06:00). Teklad LM 485 Mouse/Rat Diet 7012 (Madison, WI) and water were provided *ad libitum*.

An aqueous solution of 2.1 mg/l of sodium arsenite was prepared and 200 ml was added to plastic drinking bottles. BaP treatments were prepared in acetone so that 25 μl contained 200 nmol BaP (~2 mg/kg). BaP solutions were stored wrapped in foil at −20°C until use and were kept in an ice bath during treatment to minimize evaporation of the carrier.

Treatment groups are described in Table I. Sodium arsenite was provided to +BaP/+arsenic and −BaP/+arsenic groups *ad libitum* in drinking water for 9 days to allow the arsenic concentration to approach steady state conditions (estimated dose, 0.5 mg/kg/day). Twenty-four hours prior to topical application of test materials, mice were shaved in the upper dorsal region using electric clippers. BaP topical treatments were performed following the ninth day of arsenic pre-treatment. The +BaP/−arsenic and +BaP/+arsenic treatment groups each received BaP treatment daily for 4 consecutive days. Twenty-five microliters of acetone was pipetted topically to each −BaP/+arsenic mouse. Arsenic solution or tap water was provided *ad libitum* to the designated animals throughout the BaP topical treatments. All animals were killed by asphyxiation with carbon dioxide 24 h after the fourth daily dose of BaP. Samples of lung, skin, distal skin, spleen and urinary bladder were removed from each mouse. Tissues were stored in −80°C until DNA isolation was performed.

DNA isolation

Tissue samples were removed from −80°C and placed on ice. DNA was isolated following the instructions provided with the Wizard® kit using 30–35 mg of skin or 25–30 mg lung tissue. DNA concentrations were estimated using a Beckman DU® 640 spectrophotometer. Duplicate measurements of each sample were obtained.

^{32}P-Postlabeling analysis

^{32}P-Postlabeling was performed using nuclease P1 enhancement as described previously (19), except for minor laboratory modifications. Samples were randomized and coded prior to analysis. Adenosine triphosphate synthesis was performed as described by Johnson and Walseth (20). Specific activity was typically between 3000 and 3500 Ci/mmol. Hydrolysis, labeling, contact transfer and chromatographic conditions were as described earlier (19). After chromatography plates were air dried and placed with film in cassettes and exposed at −80°C for ~24–48 h. The film was developed and used as a template to excise adducts on each chromatogram and spots were excised from the plates and placed into liquid scintillation vials. Five microliters of 70% ethanol was added and either a Packard Tri-Carb, model 1900CA or 2200 CA, Liquid Scintillation Analyzer was used to determine radioactivity counts. Determination of relative adduct labeling (RAL) was performed as described by Talaska *et al*. (21).

Two methods of sample preparation for arsenic analysis were performed. Initial analysis was found to be too insensitive to measure reliably arsenic at the concentrations that resulted from these treatments. After a total of 10 samples from all exposure groups were analyzed destructively, the sample preparation method was modified so as to obtain a more concentrated extract. The following describes the initial methods and modifications.

Total tissue arsenic levels were determined using ICP-mass spectrometry (ICP-MS) following microwave digestion of the tissue with nitric acid. A model NES 1000 closed vessel microwave digestion system manufactured by CEM Corporation (Matthews, NC) and an Agilent 7500 ICP-MS (Palo Alto, CA) were used. A 10 ml volume of 50:50 nitric acid:water was added to the mouse skin sample (~0.05 g) in the microwave sample tube. Microwave power was increased over four steps at 5 min intervals, starting at 250, 450, 550 and ending at 650 W. Temperature and pressure limits for each of the four steps were 120, 140, 150 and 165°C and 20, 80, 120 and 170 psi. At the end of the digestion, the samples were diluted to a total volume of 100 ml and 5% nitric acid. This solution was filtered through a 0.45 μm nylon membrane filter and indium was added as an internal standard.

Specific details of subsequent method modifications are as follows. After digestion, samples were diluted to 10 ml total volume with 18 mΩ cm deionized water (final nitric acid concentration of 25%). As higher concentrations of nitric acid are not recommended because they modify the ionization efficiency of the plasma, calibrations standards were also prepared in solutions of 25% nitric acid to match the sample matrix.

Method blanks were prepared for each sample preparation technique. The method blanks showed no detectable arsenic. The parameters used in the Agilent ICP-MS included 1300 W forward power, plasma, carrier and auxiliary gas flow rates of 15.0, 1.08 and 1 l/min, respectively. Calibration curves were prepared initially in 5% nitric acid with standards ranging from 1 to 250 p.p.b.. With the method modifications, calibration curve standards were prepared in 25% nitric acid with standards ranging from 0.5 to 100 p.p.b. All regression coefficients were acceptable, with the lowest value being 0.9999. Since sample amounts were a limiting factor, duplicates were not performed.

Statistical analysis

Data were entered into a Microsoft® 2000 Excel spreadsheet and SAS® or SPSS® were used to perform statistical tests. Normality and equality of variance were examined and data were transformed as needed; group comparisons were determined by independent *t*-tests. An $\alpha < 0.05$ was used to determine statistical significance for all statistical tests. SigmaPlot® 2001 was used to generate bar graphs and correlation plots. Unadjusted and adjusted arsenic skin concentrations were plotted against skin RAL to look for bivariate correlation trends; similarly, skin RAL values were also plotted against lung RAL values. An $\alpha < 0.05$ was used to determine statistical significance.

Table I. Animal treatment groups and abbreviations

Treatment		Abbreviations		Number of mice in the group
Arsenic	BaP	Skin	Lung	
−	−	CS	CL	5
+	−	AS	AL	5
−	+	BPS	BPL	15
+	+	BPAS	BPAL	15

AL, −BaP/+arsenic, lung; AS, −BaP/+arsenic, skin; BPAL, +BaP/+arsenic, lung; BPAS, +BaP/+arsenic, skin; BPL, +BaP/−arsenic, lung; BPS, +BaP/−arsenic, skin; CL, −BaP/−arsenic, lung; CS, −BaP/−arsenic, skin.

Results

Arsenic in the drinking water had little or no apparent effect upon the health of the animals. Arsenic treated animals gained 0.1 g more weight on average than did the non-arsenic treated animals, although these animals weighed 0.4 g less on average when received (this distribution was a chance occurrence as animals were assigned to treatment groups prior to being weighed). Animals were weighed daily at approximately the same time, early afternoon.

Daily water measurements were also taken early each afternoon. Arsenic treated groups consumed slightly less water than non-arsenic treated groups (arsenic mean = 3.5 ml, standard error = ± 0.1 versus no-arsenic 3.6 ml ± 0.1); this difference was non-significant.

Arsenic tissues concentrations were estimated by ICP-MS. Unfortunately, the low dose exposures required that the entire

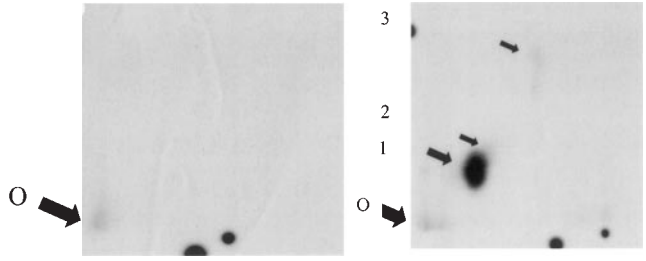

Fig. 1. ^{32}Postlabeling autoradiograms from skin DNA of animals treated with either the acetone vehicle only and no arsenic (left panel) or with BaP and arsenic (right panel) as detailed in Materials and methods. The arrows and 'O' in the figures indicate the origins for each chromatogram. The left panel shows essentially a clear field. Dark marks at the bottom are from radioactive ink for orientation and alignment. The numbered arrows in the right panel indicate the positions of the three adducts mentioned in the text. Films were exposed for ~15 h at −80°C.

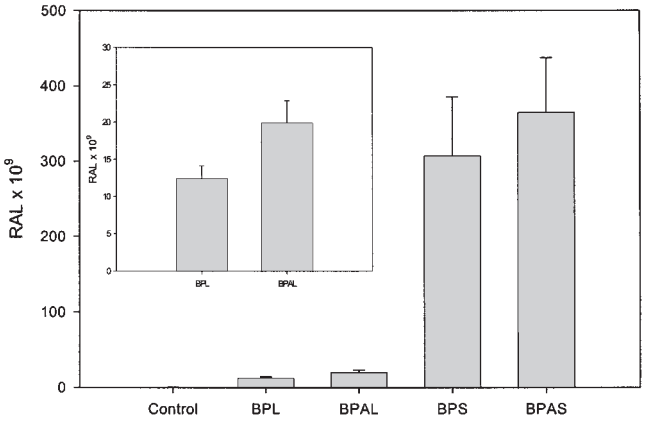

Fig. 2. Total DNA adduct levels: comparisons of the BaP versus no BaP and BaP with and without arsenic treatments in both skin and lung. Error bars are standard errors of means. Each group treated with BaP was significantly different from control group ($P < 0.05$). (Inset) Expanded scale of the BPL and BPAL groups, respectively, with the RAL × 10^9 values ranging from 0 to 50 to emphasize the difference between the two groups. The difference between these two groups was statistically significant ($P = 0.038$).

tissue sample be digested for each analysis, making duplicate determinations impossible. Initially five skin samples from animals from all groups were run using methods with a 0.2 p.p.m. limit of detection (LOD) and all these samples were at or very near the LOD. The method sensitivity was increased as described in the Materials and methods, and arsenic concentrations in the remaining treated skin tissues were measurable above the LOD. The skin arsenic concentrations in treated animals was 0.25 mg/g tissue. This level was significantly different from the background levels in the animals given tap water.

Representative autoradiograms of skin DNA samples are depicted in Figure 1. Three individual adducts could be resolved in some, but not all, skin samples, while only one adduct (adduct 1) was noted in lung tissue. For the purposes of this study, adducts were identified simply by numbers. Adduct 1 was the adduct seen in all tissues and from our previous work most likely corresponds to (+)-*trans*-anti-7R, 8S, 9S-trihydroxy-10R-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (21), although this was not confirmed by co-chromatography experiments in this study. Adducts 2 and 3 were seen only in skin samples treated with BaP. Mean adduct 1 RAL levels were 25-fold higher than adduct 2. Mean adduct 2 RAL values were 3-fold higher than adduct 3. No adducts were observed in samples from animals not treated with arsenic. It did not appear that arsenic had a differential influence on any particular adduct level. Variances for the +BaP groups were unequal and this was assumed for all *t*-test comparisons.

Both non-BaP treated control groups were combined for data analysis because they did not differ in RAL levels. The adduct levels in the +BaP groups were significantly different than −BaP controls for both skin and lung (Figure 2). Arsenic appeared to induce a shift in the relative proportion of adducts in skin and lung since mean skin BaP adduct levels in the +BaP/+arsenic group were 18-fold greater on average than in the lungs of the same animals, while adduct levels were 25-fold higher in the skin relative to the lung in the non-arsenic groups (Figure 2). Arsenic affected BaP–DNA adduct formation (Figure 2) by increasing BaP adduct levels for the same BaP dose in both lung (BPAL mean = 19.9 RAL × 10^9, standard error = ±3.0) and skin (BPAS = 364.6 ± 72.6) when compared with +BaP/−arsenic groups (BPL = 12.4 ± 1.7; BPS = 307.1 ± 78.4). A statistically significant difference ($P = 0.038$) was seen comparing the adduct levels in the lungs of the +BaP/+arsenic group and the +BaP/−arsenic group (Figure 2). However, the elevation in the RAL values induced by arsenic in the skin was not statistically significant ($P = 0.595$).

The median of the arsenic concentration in the skin of the non-arsenic treated groups (0.085 μg/g for non-arsenic treated animals) was subtracted from that of the arsenic treated animals to test for bivariate correlation trends. Skin arsenic concentrations including samples analyzed using the insensitive and sensitive procedures was not correlated with skin adduct levels ($r = -0.07$; $P = 0.796$). When the five samples obtained using the initial method were removed from the analysis a positive, but not significant, linear relationship was seen ($r = 0.537$; $P = 0.11$). Skin and lung adduct levels did not appear to be correlated within each animal, regardless of treatment.

Discussion

We report that arsenic at concentrations that produce no apparent toxicity enhances the DNA binding of BaP in a target organ, the lungs, when BaP is administered topically and arsenic is given in drinking water. The lack of toxicity of arsenic in this study is consistent with earlier low dose studies. Blakley *et al*. (22) administered 0.5, 2.0 and 10 p.p.m. of sodium arsenite and noted no decrease in weight or water consumption. Kanisawa and Schroeder (23), using a dose roughly twice as high (5 mg/l), saw no decrease in weight of the arsenic treated animals until after ~1 year.

The measurable and statistically elevated skin arsenic levels in the treated mice confirmed arsenic intake and subsequent incorporation into tissue.

Sensitivity problems hampered the accurate determination of arsenic tissue concentration in initial studies.

The chromatographic behavior of adducts in lung and skin following chromatographic separation confirms the metabolic activation and covalent binding of BaP to DNA (Figure 1). Adduct 1 for both lung and skin was located comparably with the adduct position reported by Talaska *et al*. (21), which was determined to be (+)-*trans*-anti-7R, 8S,9S-trihydroxy-10R-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,

10-tetrahydrobenzo[*a*]pyrene by co-chromatography. Adduct 2 for skin tissue appeared to be (−)-anti-7S-8R,9R-trihydroxy-10S-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene as reported by Talaska *et al*. (21). Adduct 3 in the present study was also related in position to adduct 3 reported earlier (21), although the identity of this particular BaP–DNA adduct has yet to be determined.

Higher total adduct levels in the skin compared with lung is consistent with BaP topical application (21,24,25), although this trend is not necessarily true for all PAH and mixtures (26,27). Topically applied carcinogens reach the lung via the circulation and DNA adduct levels appear to be fairly uniform throughout lung tissue. DNA adducts are seen in the skin only at the site of PAH application and not at distal sites (28). The lung contains more cytochromes P450s and other metabolic enzymes than does the skin, so activation in the lung is less likely to be saturated by high doses (29). On the other hand, if arsenic inhibits the activity of phase 2 detoxifying enzymes, such as glutathione *S*-transferase (14), then significant lung effects may be seen in spite of topical application and low tissue doses.

Maier *et al*. (14) reported that arsenic potentiated the binding of BaP to cellular DNA *in vitro*, increasing BaP–DNA adduct levels as much as 17-fold for a given BaP treatment. While the results presented here are not of the same magnitude (~2-fold increase) as reported in that earlier, *in vitro* study, arsenic does enhance BaP–DNA adduct formation in the target organ even when BaP is topically applied and the concentration of arsenic in the lung was below the limit of detection. The results from this study provide indirect support of the *in vivo* findings of Pershagen *et al*. (30) and Ishinishi *et al*. (31). Our finding of a larger relative effect of arsenic in lung tissue is interesting as a greater effect might be anticipated in the skin where the arsenic concentrations are higher (all lung samples tested in this study were below the LOD). Arsenic is thought to accumulate in keratin in the skin and keratin may serve as a high affinity site for arsenic precluding its interaction with other enzymes and macromolecules in that organ. Thus, our data suggest that the mechanism of arsenic skin carcinogenesis may be different than its mechanism in the lung. Further studies with multiple treatments and exposure levels of both materials are needed to resolve these questions.

The arsenic dose administered to animals in this relatively short-term study was within the upper range of some human environmental exposures (32–34). Chronic, occupational inhalation exposures to arsenic are permitted at a level of ~0.1 mg/kg/day (35), while in the current study the dose was estimated to be 0.5 mg/kg/day. A longer chronic exposure regimen may be necessary to achieve the steady state levels estimated in human populations and achieve maximal effects upon DNA binding.

In summary, arsenic affected BaP–DNA adduct formation by increasing RAL in both lung and skin, although only lung differences were significant ($P = 0.038$). This study supports epidemiological reports of synergistic interactions with arsenic exposure and smoking and to toxicological investigations that showed BaP and arsenic interacts to increase lung tumors; arsenic doubled the levels of BaP–DNA adducts in the lung with the same dose of BaP. These results strongly suggest that arsenic in drinking water potentiates BaP–DNA adduct formation in the lung when BaP is applied to the skin and provides a basis for a tentative explanation for arsenic and BaP interactions in the lung.

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