Benzo[a]pyrene at an environmentally relevant dose is slowly absorbed by, and extensively metabolized in, tracheal epithelium

Per Gerde¹,²,³, Bruce A. Muggenburg¹, Janice R. Thornton-Manning¹, Johnnye L. Lewis¹, Kee H. Pyon¹ and Alan R. Dahl¹

¹Lovelace Respiratory Research Institute, PO Box 5890, Albuquerque, NM 87185, USA and ²Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-17177 Stockholm, Sweden (in cooperation with the Swedish National Institute for Working Life)

To whom correspondence should be addressed

While tobacco smoke has been conclusively identified as a lung carcinogen, there is much debate over which smoke constituent(s) are primarily responsible for its carcinogenicity. Previous studies in our laboratory suggested that highly lipophilic carcinogens are slowly absorbed in the thicker epithelium of the conducting airways, potentially allowing for substantial local metabolism. The bioactivation of polycyclic aromatic hydrocarbons in airway epithelium may, hence, be important in tobacco smoke-induced carcinogenesis. In the present study, the hypothesis of slow absorption and substantial local metabolic activation of highly lipophilic carcinogen in airway epithelium was tested in dogs. A single dose of tritiated benzo[a]pyrene (BaP) dissolved in a saline/phospholipid suspension was instilled in the trachea, just anterior to the carina. At intervals of a few minutes up to 30 min over a 3-h period, blood samples were drawn from the azygous vein, which drains the area around the point of instillation, and from the systemic circulation. Tissue samples were taken at the end of the experiment. The concentration of BaP with depth into the tracheal mucosa was determined with autoradiography. BaP was slowly absorbed into the trachea with a half-time of ~73 min, which is consistent with diffusion-limited passage through the epithelium and lead to local doses in the tracheal epithelium that were more than a 1000-fold those of other tissues. The long retention of BaP in the epithelium provided the local metabolizing enzymes with high substrate levels over a long period, resulting in extensive metabolism. At 3 h after the exposure, 23% of the BaP-equivalent activity remained in the tracheal mucosa. Of this fraction, 13% was parent compound, 28% was organic extractable, 31% was water-soluble, and 28–7% of the instilled dose was bound to tracheal tissues. These results explain the tendency of highly lipophilic carcinogens, such as BaP, to induce tumors at the site of entry and, furthermore, indicate that the highly lipophilic components of tobacco smoke and polluted air may be the most important contributors to lung tumors of the conducting airways.

Introduction

In 1995, lung cancer constituted nearly one in three of all cancer deaths in the US (1), a dramatic increase from earlier in the century. Epidemiological studies linked tobacco smoking with increased lung cancer as early as 1950 (2,3), setting off an intense search to identify the most active constituents in the complex mixture of chemicals comprising tobacco smoke, and to establish a dose–response relationship. Fractionation of smoke condensate according to physicochemical properties indicated that polycyclic aromatic hydrocarbons (PAHs*) were possible culprits in causing lung cancer among smokers (4–6) because instillation exposures of rodents to large amounts of PAHs, which far exceeded the dose received by smokers, induced lung tumors (7–9). Quantitative relationships for the disposition, metabolism, and tissue binding of PAHs and other tobacco smoke carcinogens (10–12) raised doubts about the singular importance of PAHs in lung cancer, both because of the massive doses required to produce carcinogenic effects (13,14) and because PAHs require metabolic activation (15), a function that is much lower in the lungs than in liver (16,17). Careful reading of the literature reveals three implicit assumptions behind the arguments against PAHs being the major lung carcinogen in tobacco smoke: (i) The exposure–target dose relationship is approximately linear over wide ranges of exposures, which lead to the prediction that extrapolation from the massive doses needed to produce cancer experimentally would lead to minimal responses of realistic concentrations; (ii) the general shape of the dose–response curves for all classes of inhaled organic carcinogens are similar; and (iii), the systemic concentration of inhalants are similar to those at the portal-of-entry, leading to the conclusion that enhanced portal-of-entry sensitivity must stem from either inherent tissue sensitivity or high local rates of activation. Perhaps in part because of such unproven assumptions, more exact dosimetry for cigarette smoke components has been called for (18). The results reported in this communication indicate that, for highly lipophilic inhalants such as PAHs, all three assumptions may be unwarranted, and PAHs may, indeed, play a major role in the etiology of lung cancer.

Materials and methods

Test materials

To test the effect of high lipophilicity on retention in airway epithelia, we selected benzo[a]pyrene (BaP), which has an octanol/water partition coefficient of nearly 10⁶ (19). To achieve the low quantitation limit needed for a ‘realistic’ dose, i.e. one similar to that received by smokers, tritium-labelled BaP was used; [G-³H]benzo[a]pyrene, 2.33 TBq/mmol, >99% pure (Amersham, TRK.662, Batch 85). All radioactive samples were counted on a Packard Tricarb (Model 2500 TR) liquid scintillation counter (LSC) using Ultima Gold (Packard) as scintillation cocktail.

To minimize vehicle-related artifacts caused by desorption from carrier particles or dissolution of crystalline BaP (20), BaP was instilled as a solution in 61 µg l-α-phosphatidylcholine (Sigma, P5388) ml of saline (0.015 g BaP/g phosphatidylcholine). The absence of crystalline BaP was confirmed by fluorescence microscopy. The normal content of surfactant in the airway lining layer of the central airways is ~35 mg/m² (21). The surface area of the 1-cm-long tracheal segment on which the BaP was deposited was ~5 cm². Thus, the instilled phosphatidylcholine was <10% of the natural surfactant content. The test solution was prepared as follows: 0.89 µg [³H]BaP was mixed with...
with 61 µg phosphatidylcholine in 1 ml hexane. The solvent was evaporated to dryness under nitrogen. The precipitate was maintained at 40°C for 30 min, to permit thorough mixing of BaP and surfactant, and was dissolved in 1 ml saline. Protected by a stream of nitrogen and cooled in an ice bath, the solution was sonicated for 6×20 s using a probe sonicator (Model S-125, Branson Sonic Power, Danbury, CT) at power setting 2. Between each run with the sonicator, the solution was cooled for ≥20 s. Solutions were prepared fresh the day of exposure.

**Animals**

A single spray bolus of BaP was instilled in the trachea of beagle dogs about 30 mm from the carinal ridge of the main bifurcation (Figure 1). Absorption through the tracheal epithelium was measured by repeatedly sampling blood from the azygous vein draining the local area of the tracheal mucosa (Figure 1), and from both sides of the systemic circulation, the posterior vena cava, and the thoracic aorta. By measuring the differences between BaP concentration in the azygous vein blood and that in the systemic circulation, absorption in the tracheal mucosa was monitored. At the end of the 3-h exposure, tissue samples were removed for later determination of retained radioactivity.

Four beagle dogs of either gender weighing 9.4 ± 0.5 kg from the Institute’s colony were used in this study. The dogs were housed in indoor outdoor kennel runs, two dogs per run, fed 350 g of dry kibble (Teklad Mini Lab Dog Diet ST59, Madison, WI) once a day, and had water available at all times. Before each procedure, the dogs were housed briefly in cages, one dog per cage, in the Institute’s veterinary clinic.

**Verification of exposure level**

Instillations were made using the Lovelace microspray nozzle (22). In brief, a nozzle with eight radially directed orifices of 150 µm diameter was inserted into a 1.1 mm o.d. tubing, which, in turn, was passed through the biopsy channel of a fiberoptic bronchoscope (Olympus, Model BF, Type 4B2). By visually placing the bronchoscope, small liquid volumes could be sprayed radially onto a precise location on the trachea. Teflon tubing (Type VW-1, Zeus Industrial Products, Orangeburg, SC) was used for spraying to minimize adsorption of surfactant and BaP onto the tube walls. Four spray tubes and nozzles were prepared immediately before the exposure; 20.0 µl of the test solutions were injected into each spray tube using a microsyringe, and the nozzle was inserted into the tube. Three of the tubes were sprayed directly into 20 ml scintillation vials as standard samples and counted (St1–St3). The nozzles and wetted parts of the emptied tubes were placed in a second series of vials for separate counting (Ri1–Ri3). The tube used for exposure was also cut and counted together with its nozzle (Ri exp). The instilled amount of material S exp was determined according to:

\[
S_{\text{exp}} = (S_1 + S_2 + S_3 + R_1 + R_2 + R_3)/3 - S_{\text{exp}}
\]

**Animal procedures**

Food was withheld for 18 h prior to the procedure. Each dog was given 0.2 ml of acepromazine subcutaneously. Anesthesia was induced 15 min later with isoflurane using a face mask. When light surgical anesthesia was achieved, an endotracheal tube was placed in the trachea, and surgical anesthesia was maintained via the tube. Dogs were prepared for aseptic surgical procedures. A surgical cut-down procedure over the femoral artery and vein was done, and catheters filled with heparinized saline were passed into and positioned, respectively, in the posterior vena cava close to the right heart and in the thoracic aorta. The right chest was entered through the intercostal space. The apical and cardiac lung lobes were pushed posteriorly, and the azygous vein was located. The vein was dissected free, and a blood flow meter was positioned around the vessel 3–4 cm from the azygous vein anterior vena cava junction. Then a heparin-saline filled catheter was passed into the vein lumen between the flow meter and the anterior vena cava junction to collect blood periodically. Blood flow through the azygous vein was monitored using a Doppler ultrasound flowmeter (Triton Technology, System 6, Model 206, San Diego, CA).

**Exposure and monitoring**

From the moment of instillation, blood was sampled from the catheter inserted in the azygous vein and from those in the aorta and posterior vena cava for 3 h. Samples of 1 ml were obtained for determination of total radioactivity, and 10 ml samples were obtained for HPLC determination of the metabolite pattern in the blood. Immediately after collection, blood samples for combustion were placed in 2-ml paper thimbles (Packard Instruments, Meriden, CT) filled with cotton for use as a combustion aid. The samples were placed in dry ice, then stored at −20°C until combustion.

**Post-exposure**

The isoflurane concentration was increased to provide deep surgical anesthesia; the dogs were then killed by exsanguination through a heart puncture. During this procedure, the area proximal to the cuff of the tracheal catheter was lavaged with 3×30 ml saline to collect mucous-retained materials that may have accumulated during the exposure period. Immediately after death, the trachea, the extrapulmonary bronchi, and lungs were excised intact from the thoracic cavity. Using an airway dissection technique (23), the tracheal and bronchial segments that may have been exposed were microdissected into 1-cm-long segments starting from the larynx, proceeding through the left main bronchi down to the fourth generation and through the right main bronchi down to the fifth generation. Circulation had ceased 10–15 min after beginning the exsanguination, and the tracheobronchial tissues were frozen within another 15–20 min. Three tracheal segments around the point of instillation were set aside for extraction and HPLC fractionation of metabolites and for determination of bound radioactivity. Samples were also taken of the lung parenchyma, heart, liver, kidney, small intestines, gall bladder content, skeletal muscle, urine, adipose tissue, and blood. All samples were stored at −20°C.

**Determination of radioactivity in tissues**

Prior to determination of radioactivity by combustion, samples were dried on a vacuum line for 2–4 h, the distillate being collected in a cold trap filled with liquid nitrogen (24). Tritiated water in the aqueous distillates was determined by LSC. Tests verified that negligible amounts of the labelled BaP were used in this study. The dogs were housed in indoor outdoor distillation during the drying procedure. The change in specific activity of organic metabolites from loss of tritium to water was not corrected.

To determine non-volatile tritium content of blood and tissue, samples were oxidized using a combustion method modified from that of Knoche and Bell (25). Details of this procedure will be published elsewhere (Gerde et al., in preparation), but the major change was to redesign the gas inlets so that stable combustion could be achieved without using porous plugs in the tube furnace. This modification increased the fraction of 3H collected to almost 100%. In addition, a cold trap with liquid nitrogen was used to retain the entire flow of combustion gases from the furnace.

**Fractionation of metabolites and high performance liquid chromatography**

The tissue samples and the ~10 ml blood samples for analysis of metabolite patterns using HPLC were stored under argon at −80°C until used. Blood samples were dried on the vacuum line as described above. The residue was dissolved/suspended in 15 ml saline. The suspensions were extracted with 5×15 ml ethyl acetate with periodic sonication, and the organic fraction was removed. The aqueous fraction was centrifuged, and the supernatant and separated pellet were combusted separately and counted by LSC. The pooled organic fractions contained lipids which reduced the uptake of BaP and itsof vials for separate counting (Ri1–Ri3). The tube used for exposure was also cut and counted together with its nozzle (Ri exp). The instilled amount of material S exp was determined according to:

\[
S_{\text{exp}} = (S_1 + S_2 + S_3 + R_1 + R_2 + R_3)/3 - R_{\text{exp}}
\]
tion of total radioactivity. The remaining aliquot was dried on the vacuum line to remove tritiated water. After addition of 4 ml of saline, this aliquot was extracted with 5×5 ml ethyl acetate/acetone (2:1). The aqueous fraction was centrifuged at 1440 g to separate water-soluble metabolites from the tissue pellet. The pellet and triplicate aliquots of the aqueous supernatant were combusted to determine the fraction of tissue-bound and water-soluble metabolites, respectively. The organic fraction was treated as described above for the blood samples.

HPLC analysis was carried out on a Spectra-Physics 8100 instrument using a Ultrasphere C18 5 µm×25 cm×4.6 mm column (Beckman) after injecting the sample in 150–250 µl of MeOH. A ramp gradient from 55% MeOH/H2O to 100% MeOH in 30 min at 1 ml/min was used.

 Autoradiography

To determine the toxicant distribution with depth into the mucosa by autoradiography, BaP was instilled in a single animal, which was then killed in order to have the tracheal tissues of interest frozen at approximately the half-time (Figure 2) of BaP in the mucosa. To obtain sufficient activity, the amount of instilled BaP was increased 9-fold to 104 ng; the amount of surfactant was proportionally increased in order to solubilize the entire amount of the PAH, but the instilled volume of saline was kept at 20 µl. Blood was sampled only from the right heart for 30 min after instillation. No tracheal lavage was done at the end of the exposure. At 30 min after instillation of BaP, anesthesia was increased to achieve deep surgical level and exsanguination by cardiac puncture started. Circulation ceased at 45 min after the instillation. The trachea and main-stem bronchi were sampled as done with the three other dogs. The excised trachea was microdissected into 5-mm long segments; alternating segments were combusted directly to determine concentration of radioactivity or embedded into Optimum Cutting Temperature (Sakura Fine Tek, Torrance, CA) embedding medium and mounted frozen on chucks for autoradiography. The tracheal segments for autoradiography were frozen in liquid nitrogen for 60 min after the instillation and were stored at ~18°C until sectioned. From the segment containing the highest radioactive concentration, as determined by combustion of adjacent segments, 10 µm-sections of the entire tracheal ring were cut at ~18°C into whole rings, mounted on glass slides, and placed in a light-proof, lead-lined cassette. The sections were exposed to Fuji-blue film; cassettes were sealed and stored at ~20°C for 3 or 6 weeks. In addition to autoradiography, alternate serial frozen sections were stained with hematoxylin and eosin to obtain accurate morphometry of the tracheal mucosa. The thickness of the epithelium, the measured perpendicular from lumenal-to-basement membrane, was determined at 20 evenly spaced positions around the tracheal circumference in three different dogs.

Densitometry was used to quantitate the depth distribution of the radioactivity in the mucosa on the autoradiographs, and was conducted using the NIH Image (v 1.55) public domain software on a Macintosh Quadra 950 computer, coupled to an Olympus BH-2-RFCA light microscope (Olympus Corp, Lake Success, NY) equipped with an Opti-Quip 12 volt DC regulated constant light source (Opti-Quip, Inc., Highland Mills, NY). The radioactivity was distributed primarily into two different levels: one higher in the epithelium and one considerably lower in the adjacent subepithelium. Therefore, only the relative level of radioactivity in the epithelium and subepithelium was determined at a region of the autoradiograph showing a clear and strong distribution of radioactivity. Using a 20-µm diameter measuring window to account for any variability in density, 20 adjacent measurements were made along the autoradiograph signal that corresponded to the center-line of the epithelium. The same circular window was also used to make 20 adjacent measurements in the subepithelium along the region parallel to that measured in the epithelium. The radial distance from the center of the circular window in the subepithelium to the basement membrane of the epithelium was ~20 µm. In this paper, the term subepithelium refers to all structures between the basement membrane and the tracheal cartilage.

Results

To determine the rate of penetration of BaP through airway epithelia, a site was required from which the efficient blood was not seriously diluted by blood draining from other tissues. Such a site occurs in the trachea of the dog (26). Test instillation using 111In-labeled EDTA (data not shown) confirmed that the extrapulmonary parts of the canine bronchial tree were to a large extent drained through the azygous vein. In addition, if even a small fraction of the instilled BaP were to reach the alveolar region, from which clearance is rapid (27), the slow clearance from the azygous vein would be obfuscated by the BaP in the systemic blood.

Our primary goal was to study the absorption of a realistic dose of BaP through the tracheobronchial mucosa; our reference was the amount of PAHs deposited on the airway surface of a human smoking one cigarette (Table I). Based on body weight, the amount of BaP administered was within the range that is deposited by smoking one cigarette; based on unit area of the exposed airway surface, the experimental dose was about two orders of magnitude higher than the peak concentration likely to be encountered in a smoker’s lungs but was within an order of magnitude of that for the total PAHs.

Disposition

The rate of absorption of BaP was indicated in three ways: (i) the changes in concentration of BaP in azygos vein blood;
Table I. Comparison of the amounts of BaP instilled in the dog with the estimated amounts of BaP and total PAHs deposited in humans after smoking one reference cigarette (28)

<table>
<thead>
<tr>
<th>Experimental exposures in dogs:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP administered</td>
<td>12.0 ± 0.3 ng</td>
<td>(46.7 ± 1.2 pmol)</td>
</tr>
<tr>
<td>Density of deposition</td>
<td>1400 ng/m²</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Smoking one reference cigarette in people:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP content in condensate</td>
<td>10–50 ng (40–200 pmol)</td>
<td></td>
</tr>
<tr>
<td>Density of deposition of BaP: average</td>
<td>0.2 ng/m²</td>
<td></td>
</tr>
<tr>
<td>Density of deposition of BaP: peak</td>
<td>6 ng/m²</td>
<td></td>
</tr>
<tr>
<td>Sum of 21 PAHs in condensate</td>
<td>1000 ng</td>
<td></td>
</tr>
<tr>
<td>Density of deposition of 21 PAHs: average</td>
<td>10 ng/m²</td>
<td></td>
</tr>
<tr>
<td>Density of deposition of 21 PAHs: peak</td>
<td>300 ng/m²</td>
<td></td>
</tr>
</tbody>
</table>

Surface deposition during the exposures was based on the approximate surface area of the entire trachea distal to the inflatable balloon of the tracheal catheter. In human smokers, the average surface density of deposition was based on a 50% deposition of the smoke condensate (29) deposited over 81 m² surface area of the lungs (30). The peak density of deposition in human smokers was assumed to be 30 times the average density of deposition in the lungs (21).

(ii) the changes in concentration of BaP in systemic blood; and, (iii) the retained BaP in the airway mucosa at the end of exposure.

BaP-derived tritiated compounds appeared slowly in the azygous vein blood, peaking at ~40 min after exposure. Samples drawn from the systemic circulation 2 min after instillation had low levels of radioactivity, indicating that very small amounts of the sprayed bolus had deposited in and cleared always from the alveolar region. Radioactivity in the azygous vein, relative to that in the systemic blood, was highly variable but was always substantially greater than that in systemic blood (Figure 2). The variability may have been caused by a substantial fraction of the instilled BaP being transported in mucus to the laryngeal end of the trachea, and from there being absorbed into blood vessels other than the azygous vein, as this area is also drained by other veins (31).

Throughout most of the exposure, blood sampled from the right heart, which was upstream of the flow convergence with blood from the azygous vein, had a lower concentration than downstream blood sampled from the left heart. The concentration in the systemic circulation showed a continuous increase throughout almost the entire exposure, indicating that net systemic elimination of BaP had barely started at 3 h post-instillation.

The distribution of BaP equivalent (BaP-eq) activity along the luminal surface of the upper bronchial tree 3.2 h after the instillation is shown as an average for all three dogs in Figure 3. The influence of mucociliary clearance is evident from the high activity collected in the anterior part of the trachea, from which further movement was prevented by the inflated cuff of the endotracheal tube. The high surface concentration at the inflated cuff was contributed almost entirely by dog C, which also had the lowest concentration in azygous vein blood. At 3 h after the instillation the mucus was lavaged from the trachea: little radioactivity remained (Table II). This is consistent with a previously reported 10 min half-time of BaP in the mucus (32). The low content of BaP in the mucus also indicates that, at that point, the BaP had desorbed from its carrier liposomes, because these were likely to have remained in the mucus (33,34). The surface concentration of BaP distal to the point of instillation dropped rapidly, indicating limited spread of the aerosol from the point of instillation. This is consistent with very little radioactivity in the right heart blood 2 min after instillation, because aerosol deposited distally, in part, would have reached the alveoli and would have been rapidly absorbed into the pulmonary circulation.

A total of 23% of the instilled radioactivity was in the tracheobronchial region at the end of exposure (Table II); of this, 72% was in the form of soluble metabolites and parent compound (Figure 4). This soluble fraction of BaP-eq, thus, had an average half-life of retention during the first 3.2 h of ~73 min. In tissues distal to the lungs, the highest concentrations of BaP-eq after 3.2 h were in the liver and kidney (Table II). The lowest concentration was in the adipose tissue. The low concentration in adipose tissue, despite the high lipophilicity of BaP, may result from a combination of low blood perfusion of this tissue and slow diffusion of BaP across the aqueous barriers separating the blood from the fatty tissue. Despite the slow transport, some unmetabolized BaP does eventually accumulate in the fat (36). The tissues in which radioactivity was measured constituted ~65% of the total body mass, and contained 59% of the total instilled radioactivity (Table II).

 Autoradiographs

Most of the radioactivity retained in the trachea was in the epithelium; there was a sharp drop in concentration at the basement membrane (Figure 5). Based on densitometry measurements, ~73% of the radioactivity was retained in the 23 ± 9-µm (SD, n = 3) thick epithelium; most of the remaining activity was retained within 70 µm of the basement membrane. This information was combined with the total radioactivity derived from combustion of tracheal segments to assess the epithelial dose of BaP (Table II). The concentration retained in the epithelium at the end of exposure, as based on the average surface concentration in the exposed parts of the trachea (Figure 3), was thus 42 000 fmoL BaP-eq/g, assuming a tissue density of 1 g/cm³ (Table II). This level is ~2200-fold that in the liver, the distal tissue was the highest concentration of BaP-eq. In addition to the low concentration in the subepithelium, small amounts of radioactivity were found in the duct cells of the submucosal glands (Figure 5).
Dosimetry of BaP in airway mucosa

Table II. Tissue distribution of BaP and metabolites and water-associated tritium 3.2 h after instillation

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total tissue weighta (g)</th>
<th>Tritium associated with waterb (% of instilled)</th>
<th>BaP and organic metabolitesb (% of instilled)</th>
<th>Conc. of BaP and organic metabolites in wet tissueb (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trachea + bronchi</td>
<td>19.7 ± 2.0b</td>
<td>0.2 ± 0.1</td>
<td>23 ± 10</td>
<td>570 ± 320</td>
</tr>
<tr>
<td>Tracheal epithelium</td>
<td>0.20</td>
<td>—</td>
<td>17</td>
<td>42 000</td>
</tr>
<tr>
<td>Tracheal lavage</td>
<td>—</td>
<td>0.08 ± 0.02</td>
<td>1 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>Peripheral lung</td>
<td>69</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>3.5 ± 1.4</td>
</tr>
<tr>
<td>Liver</td>
<td>250</td>
<td>0.3 ± 0.1</td>
<td>10 ± 2</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Gall content</td>
<td>11 ± 3b</td>
<td>0.04 ± 0.03</td>
<td>7 ± 3</td>
<td>290 ± 120</td>
</tr>
<tr>
<td>Kidney</td>
<td>42</td>
<td>0.04 ± 0.01</td>
<td>0.5 ± 0.1</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td>Urine</td>
<td>—</td>
<td>—</td>
<td>0.34 ± 0.02</td>
<td>89 ± 56</td>
</tr>
<tr>
<td>GI tract</td>
<td>390</td>
<td>0.4 ± 0.1</td>
<td>2.3 ± 0.7</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Fat</td>
<td>470</td>
<td>0.03 ± 0.01</td>
<td>0.2 ± 0.04</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>Muscle</td>
<td>4300</td>
<td>3.5 ± 1.1</td>
<td>13 ± 3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Heart</td>
<td>72</td>
<td>0.09, 0.07</td>
<td>0.16, 0.11</td>
<td>1.05, 0.65</td>
</tr>
<tr>
<td>Blood</td>
<td>610</td>
<td>0.5 ± 0.1</td>
<td>1 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Total</td>
<td>6200</td>
<td>5.1 ± 1.6</td>
<td>59 ± 11</td>
<td>—</td>
</tr>
</tbody>
</table>

aTrachea + bronchi and gall content were weighed; weight of tracheal epithelium was estimated here, and remaining tissue weights were from Cuddihy et al. (35).
bMeans of three dogs ± SD. Results for heart tissue from dogs B and C, respectively, are shown individually.

Metabolism

Metabolism is an important prelude to the clearance of BaP from the tracheobronchial mucosa. Non-metabolizing tissues spiked with BaP in vitro did not produce appreciable amounts of tritiated water, indicating that tritiated water in the tissues was generated primarily from metabolic oxidation. Diffusion and metabolism in the tracheal mucosa did not stop until tissue specimens were frozen 15–20 min after circulation had ceased. During this period some molecules that normally would have rapidly diffused into blood, primarily tritiated water and conjugated metabolites, accumulated in the tracheal tissues. Except for these artifactually high concentrations in the trachea, high levels in gall, and low concentrations in adipose tissues, the tritiated water was distributed evenly throughout the body (Table II). Based on the concentrations measured in all tissue samples, it was estimated that, at the end of the exposure, tritiated water constituted ~5% of the instilled amount of radiolabel. By not correcting for this partial loss of radiolabel from metabolites, the fraction of BaP-eq that was parent compound was slightly overestimated.

The radioactivity of the organic-soluble fractions from the systemic blood was not high enough to allow fractionation on the HPLC. In azygous vein blood from dog B, the fraction of BaP that appeared as parent compound was highest in the first sample 20 min after instillation, and constituted ~55% of the total (Figure 6). Concentration of parent compound dropped continuously to ~2% at 3 h. Water-soluble metabolites, making up most of the remaining radioactivity, increased from 21% to 85% during the 3 h period. The fraction of activity that was bound to blood proteins was 10–20%. Only 1–8% of the radioactivity in the blood was organic extractable metabolites.

At the end of exposure (Figure 4A), 13% of the tracheal radioactivity was associated with parent compound, and 28% was organic extractable metabolites, mostly BaP-diones (Figure 7). Radioactivity associated with water-soluble metabolites constituted 31%, with the remaining 28% covalently bound to tissues; thus, ~7% of the total instilled radioactivity was bound in the trachea. At the end of exposure, the relative concentration of water-soluble metabolites in azygous vein blood was markedly higher than in the tracheal tissues (Figure 4).
P. Gerde et al.

gens in the conducting airways would provide the local activating enzymes of the lining epithelium with high substrate levels over a long period, which could lead to a high percentage of the parent compound being metabolized even at low enzyme levels (38, 39). If metabolism is attended by activation, the high dose of activated carcinogens in bronchial epithelial cells could contribute substantially to the high frequency of tumors originating in the bronchial/bronchiolar epithelia of smokers (40).

The absorption of BaP in the tracheobronchial mucosa, as determined in the present study, is consistent with slow diffusion of this highly lipophilic compound through the epithelium. This was manifested in three ways: (i) the prolonged retention of BaP in the mucosa; (ii) the concentration gradient of BaP with depth into the mucosa; and (iii) local metabolism of BaP in the tracheal epithelium. All three phenomena are driven primarily by one physico-chemical mechanism: the transport of lipophilic diffusants in a lipid/aqueous heterogeneous medium. Cells can be viewed as lipid vesicles dispersed in a more or less continuous aqueous phase. Partitioning of lipophilic diffusants into the membranes decreases the molecules available for diffusion through the aqueous gaps between the cells, resulting in markedly slowed diffusional transport. This phenomenon will be most dominant at the site-of-entry of the diffusant into the body. The lipophilic substance dissolves readily in the membranes of the first cell layer encountered, that is, in the epithelium, but is transported slowly into the next layer. As soon as the circulation is reached in the capillary bed, the immense transport capacity of the blood will rapidly dilute the absorbing substance to the low levels at which all distal tissues are exposed. The result is a highly localized dose to the lining epithelium that has often either been overlooked or confused with artifactual vehicle effects in various experimental models (20).

Inhaled BaP has a markedly bimodal clearance from the lungs following an inhalation exposure. In the first phase, material deposited on the alveolar air/blood barrier is rapidly cleared. In the second phase, material deposited on the thicker epithelia of the conducting airways is slowly cleared. In the dog, the half-time of clearance of BaP from the alveoli is ~2.3 min (27), whereas, as determined in this study, the half-time of absorption from the trachea is ~73 min. The half-time of absorption in the tracheal mucosa is consistent with the 80-min half-time of undifferentiated, total radioactivity reported earlier (32) and the 100-min half-time for metabolized BaP predicted by a mathematical model (37). The maximum clearance rate from the trachea occurred at ~40 min after instillation, compared with some 50 s for alveolar clearance (39). Absorption in the present study was measured in the trachea, which is not often the site of tumors. However, because of qualitative similarities to the epithelia of the smaller airways, the basic principles of dosimetry found can confidently be extrapolated to the bronchial/bronchiolar airways where lung cancer more commonly arises.

The full impact of prolonged retention of highly lipophilic substances in the mucosa of lining epithelia on toxicity is revealed by the distribution of the substance with depth in the mucosa. Virtually the entire delay in absorption is a result of slow passage through the epithelium, resulting in a very high dose in this target layer of cells. Thus, most of the tissue resistance to diffusion lies between the basolateral membranes of the epithelial cells across the basement membrane to the most superficial capillaries of the subepithelium. The relatively
rapid diffusion within the epithelium most likely results from near continuity of the membranes in this cell layer. There is less such continuity in the subepithelium.

Prolonged retention of BaP in the tracheobronchial epithelium will influence the biochemical and molecular events leading to cancer. The most relevant dose for lipophilic procarcinogens, such as BaP, is likely to be the level of activated metabolites produced in or near target cells. A total of 90% of the radioactivity retained in the tracheal epithelium at the end of a 3-h exposure (Figure 4A) was associated with metabolites of BaP, which probably were generated there: relocation from other tissues against at least a 2200-fold concentration gradient is impossible without active transport, which is highly unlikely in this case. The prolonged retention of lipophilic substrates in the epithelium provides the opportunity for substantial metabolic conversion despite low enzyme activities (41). The distribution of BaP-eq in the mucosa (Figure 5) is similar to the distribution of cytochrome P450 and glutathione transferase (42,43). The metabolizing enzymes, thus, are localized where they are likely to be most effective for metabolizing exogenous, highly lipophilic substrates to less lipophilic, more easily cleared metabolites, thereby preventing accumulation of membrane-perturbing levels of substrate.

Neoplasia may be an unfortunate byproduct of this defense mechanism.

The bimodal clearance of highly lipophilic procarcinogens in the lungs is likely to carry over into a bimodal pattern of metabolic activation, both local and systemic. The local metabolism mostly occurs in the epithelium of the conducting airways; BaP deposited in the alveolar epithelium clears rapidly to the blood, most likely as parent compound. This systemic fraction of the dose will be metabolized in the liver. Because systemically generated metabolites are likely to dominate blood levels after exposure to BaP-containing aerosols, metabolites as biomarkers of exposure will be poor indicators of dose to the airway epithelium.

The distinctive depth distribution of BaP-eq activity in the airway mucosa suggests that calculations of relevant dose, that to airway epithelium, should be based on the weight of epithelium only. The primary reactive metabolite thought to be responsible for genotoxicity, BaP-7,8-diol-9,10-epoxide, is relatively lipophilic (44), indicating that it, like BaP, will be retained in the epithelium for a prolonged period. The high levels of genotoxicant probably result in much more genetic damage in tracheobronchial epithelium than in any other tissue, which could explain the mounting evidence that reactive metabolites of BaP and similar compounds are involved in transformation in human lung (45). Studies of the effects of polymorphism of metabolizing enzymes on susceptibility to lipophilic procarcinogens may give clearer results if the investigations target the enzyme expression in the tracheobronchial epithelium, rather than non-target cells in blood or elsewhere.

An important use of mechanistic knowledge of the microdistribution of inhaled carcinogens in the airway mucosa is to explore non-linear relations between exposure level and dose to airway target cells. Such effects are likely to greatly impair high to low dose extrapolations that are necessary for use in cancer risk assessments. BaP that penetrates the mucous-lining layer is rapidly absorbed in the epithelial cells, but is slowly transported into the adjacent capillary bed. Therefore, the epithelium attenuates comparatively high concentrations even at environmental exposure levels, and, as the levels are raised much beyond environmental levels, epithelial capacities to dissolve and metabolize BaP become saturated. Unaccounted for in an experimental exposure scenario, these limiting capacities may induce drastic non-linearities between exposure and response. A likely result for highly lipophilic procarcinogens is that risk assessments based on animal experiments conducted at exposure levels greatly exceeding these saturation points, may greatly underestimate cancer risk in humans following decades of environmental exposures.

Acknowledgements

This research was sponsored by NIH Grant RO1-5910 from NIEHS in facilities belonging to the US Department of Energy, Office of Health and Environmental Research under Contract DE-AC04-76EV01013, with contributing funding from the Swedish Council for Working Life Research, Grant 91-0359. The work was conducted in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. The authors acknowledge the skilful assistance of several members of the staff in this project; Dr Mary Berry and Ms Marjorie Billau, with the surgical procedures; Ms Rhonda Garlick and Mr Dean Kracko in pathology; and Mr Tony Stephens, Mr Gary Scott, Ms Margo Allen, and Mr Kevin Rohrbacher with the chemical analysis.

References


