Metabolism and Covalent Binding of $[^{3}H]$Benzo(a)pyrene by Isolated Perfused Lungs and Short-Term Tracheal Organ Culture of Cigarette Smoke-exposed Rats

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SUMMARY

The metabolism and covalent binding of the environmental carcinogen benzo(a)pyrene, a constituent of cigarette smoke, has been determined in isolated perfused lungs of sham- and cigarette smoke-exposed and 3-methylcholanthrene-pretreated male Wistar rats. Rats were exposed to cigarette smoke for 1 hr daily for either 1 or 10 days. Benzo(a)pyrene (2 nmoles) was instilled intratracheally, and metabolites in lungs and a nonrecirculating perfusate were determined. Benzo(a)pyrene was metabolized by isolated perfused lungs of sham-, cigarette smoke-exposed, and 3-methylcholanthrene-treated animals to ethyl acetate-soluble metabolites, which cochromatographed with 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, and 3-hydroxybenzo(a)pyrene. An unidentified metabolite (Y) migrating between 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene was observed in perfusates from lungs of animals that had been exposed either to cigarette smoke or 3-methylcholanthrene. Metabolite production was significantly increased by exposure to either cigarette smoke or 3-methylcholanthrene. Tracheas of sham and smoke-exposed animals also converted benzo(a)pyrene to metabolites that cochromatographed with reference dihydrodiols and 3-hydroxybenzo(a)pyrene; however, no increase in metabolite production was observed in the smoke-exposed animals. Dihydrodiols were the major ethyl acetate-soluble metabolites formed by the trachea, whereas 3-hydroxybenzo(a)pyrene was the major metabolite formed in lung perfusions. After intratracheal instillation of $[^{3}H]$benzo(a)pyrene, the amount of covalently bound radioactivity was significantly increased in the lungs of animals exposed to cigarette smoke compared to the corresponding sham-exposed animals.

INTRODUCTION

Lung cancer is the major cause of male cancer deaths in many countries. Although cigarette smoking is one of the major risk factors in the development of lung cancer, environmental factors are also of importance (7). PAH, such as BP, are both constituents of cigarette smoke and environmental pollutants. Because these agents are potent carcinogens of the respiratory tract in animals, their role in the etiology of lung cancer in man is under continuing investigation. The BP content of cigarettes (0.2 to 12.25 µg BP per 100 cigarettes) is possibly insufficient to account for the large number of cancer deaths (34). However, cigarette smoke also contains other irritants, ciliostatic agents, tumor promoters, and initiators, all of which may modify the response of the respiratory tract to BP. Other agents in cigarette smoke have also been implicated in the rise in lung cancer deaths, e.g., nitrosamines, arsenic, and polonium.

Since most chemical carcinogens have to be metabolically activated in the body before exerting their carcinogenicity (19), it is important to study the metabolic fate of carcinogens, particularly in their target tissues. PAH are metabolized by the microsomal mixed-function oxidase system to reactive epoxides which can then: (a) be converted by the microsomal enzyme epoxide hydratase to dihydrodiols, (b) combine with glutathione to give a conjugate; (c) rearrange spontaneously to give a phenol, or (d) attack a cellular macromolecule in what may be the toxic reaction (15, 24). However, recent evidence has shown that certain dihydrodiols may be further metabolized by the microsomal mixed-function oxidase to diol-epoxides, for example the conversion of 7,8-dihydriodiol to 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene 9,10-oxide (25). These diol-epoxides, which are highly mutagenic, may be the ultimate carcinogenic form of benzo[a]pyrene in some tissues (8, 14, 17, 18, 20, 25, 33).

Cigarette smoke exposure of animals has been shown to...
increase AHH activity, an enzyme metabolizing BP, in lung microsomes and homogenates (1, 2, 13, 21, 26, 30, 32). The pulmonary alveolar macrophages of humans exposed to cigarette smoke also have increased levels of AHH activity (4). Other studies have demonstrated the covalent binding of BP to human bronchial mucosa in organ culture (11), and more recently, Grover et al. (10) have shown that it is the 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene 9,10-oxide that is bound in such cultures. In view of the possibility that dihydrometabolites, in particular 7,8-dihydrodiol, may be the proximate carcinogen derived from BP, measurement of the metabolite profile should be of value in determining the role, if any, of BP metabolism in the etiology of lung cancer.

This was felt to be of importance since the majority of earlier studies had measured only AHH activity of lungs, which measures predominantly the production of phenols, mainly 3-OH-BP. In other studies, in order to maintain the structural integrity of the lungs and therefore more closely approximate the in vivo situation, the metabolite profile of BP by isolated perfused lungs has been studied (3, 6, 28, 29).

Since the majority of lung cancers in man arise from a smoking habit, the role, if any, of BP metabolism in the etiology of lung cancer, may be important. BP is the major constituent of cigarette smoke, and since the rodent trachea is histologically similar to the human bronchus, the metabolism of BP in short-term organ cultures of hamster and rat tracheas has been studied (6).

In this study we report the metabolite pattern and covalent binding of BP in isolated perfused lungs and short-term tracheal organ culture of sham- and cigarette smoke-exposed rats. These results were also compared to those obtained from similar experiments using lungs from animals pretreated with 3-MC. Exposure of animals to cigarette smoke and 3-MC caused significant increases in both the metabolites produced and the covalent binding of [3H]BP or its derivatives to the lungs of exposed animals.

MATERIALS AND METHODS

[3H]BP (specific activity, 20 Ci/mmmole) was obtained from the Radiochemical Center, Amersham, England, and was further purified by the method of De Pierre et al. (9). BP was obtained from Koch-Light Laboratories Ltd., Buckinghamshire, England. 3-MC and bovine serum albumin (Fraction V, Lot 46C-0253) were obtained from Sigma Chemical Co., St. Louis, Mo. Leibovitz L-15 medium and fetal calf serum were purchased from Grand Island Biological Co. Bio-Cult Ltd., Paisley, Scotland. Precoated TLC aluminum sheets coated with Silica Gel 60 (0.25-mm thickness) were obtained from Merck, Darmstadt, Germany. The unlabeled reference compounds, 4,5-dihydrodiol, 7,8-dihydrodiol, 9,10-dihydrodiol, and 3-OH-BP were either prepared by the method of Sims (23) and their identity confirmed by comparing their UV spectra with those reported in the literature or were the generous gift of Dr. H. V. Gelboin (National Cancer Institute, Bethesda, Md.).

Animals. Adult male Wistar albino rats (weighing 270 to 340 g) were used. After 8 weeks of age the animals were kept in cages with stainless steel mesh bottoms and were allowed food and water ad libitum. The rats were exposed daily to the smoke of 5 commercial filter cigarettes (containing 1 mg nicotine and 16 mg tar per cigarette, as quoted by the manufacturer) during 1 hr as previously described (26). Rats were exposed to smoke for either 1 or 10 days and sacrificed 20 hr after the last exposure. Control or sham-exposed rats were exposed to a similar air flow in an identical chamber, but the cigarette was replaced by a filter. Some animals were treated with 3-MC (20 mg/kg in corn oil) for 3 days.

Isolated Perfused Rat Lung. The isolated lungs were perfused by the method of Hartlia et al. (12) with minor modifications. The rats were anesthetized with i.p. sodium pentobarbitone (50 mg/kg), and a tracheotomy was performed. The lungs were connected to a pressure-regulating respirator (Bird® Mark 8, Bird Corp., Palm Springs, Calif.), and ventilation with air was begun (maximum positive pressure, 18 cm H2O). The thorax was opened, 500 IU of heparin were injected into the right ventricle, and the pulmonary artery was cannulated. The left ventricle was excised, and the lungs were removed and perfused with 4.5% bovine serum albumin in Tyrode's solution with glucose (22 mm) at a flow rate of 10 ml/min. The perfusion medium was gassed with 5% CO2 and 95% O2 to keep the pH within physiological levels. After an equilibration period of 3 min, 2 nmoles [3H]BP (20 µl [3H]BP in acetone + 80 µl 0.9% NaCl solution) were instilled intratracheally, and the nonrecirculating perfusate was collected in 2-min fractions for a period of 8 min. The use of such a nonrecirculating perfusate minimized further metabolism of the primary metabolites.

In preliminary experiments with untreated animals, the distribution of the radioactivity between perfusate and different areas of the isolated perfused preparation was checked after the intratracheal administration of 2 nmoles of [3H]BP. Most of the radioactivity [60.1 ± 2.2% (S.E.)] was found in the lungs at the end of 8 min perfusion, whereas 26.1 ± 4.3% of the radioactivity appeared in the medium, 12.7 ± 1.7% remained in the trachea, and 1.2 ± 0.5% was found in the heart and thymus combined (all results expressed as mean ± S.E. of 3 determinations). In some experiments large amounts of radioactivity appeared in the perfusate in the 1st 2 min. These experiments were excluded since the lungs were probably damaged by the intratracheal instillation.

Analysis of Lung and Perfusate. At the end of the perfusion, the lungs were removed and immediately placed in 4 ml of ice-cold 1.15% KCl, blotted dry, weighed, and then homogenized in an Ultraturrax with 4 volumes of 1.15% KCl. The radioactivity of aliquots of the homogenate, perfusate, and other tissues were determined using a Packard Model 306 Sample Oxidizer (modified by Dr. Niilo Kaartinen from the University of Turku; details of modification to be published). The perfusates collected during the 1st and last 4 min of the perfusion were extracted with 2 × 1 volume ethyl acetate, shaking vigorously for 40 sec on both extractions. Greater than 94% of the radioactivity was ethyl acetate soluble following similar extractions of perfusate to which [3H]BP or its metabolites had been added. Three ml of the lung homogenate were also removed and extracted with 2 × 6 ml of ethyl acetate. The ethyl acetate extracts were combined and dried with Na2SO4. Aliquots of the extracts were evaporated to dryness with N2 and dissolved in ethyl acetate, containing the unlabeled reference metabolites and BP. These were applied to a TLC plate and developed
in a mixture of benzene:ethanol (9:1, v/v), and the spots were located by inspection of the wet plates in UV (λ<sub>max</sub> = 380 nm). The metabolites of BP were quantified by cutting the chromatograms into 5-mm segments carrying the individual metabolites and the intermediate areas, and the radioactivity was counted. The percentage radioactivity from the corresponding areas of appropriate controls was subtracted from the experimental determinations before calculation of metabolite production. In most experiments good separation of the dihydrodiols was obtained; however, slight contamination of one dihydrodiol with another cannot be completely excluded. The value of total diols has been obtained from the radioactivity cochromatographing with 9,10-dihydrodiol, 7,8-dihydrodiol, and 4,5-dihydrodiol, including intermediate areas up to and including a region on the TLC plate 10 mm beyond 4,5-dihydrodiol, where a band containing significantly greater radioactivity than that in controls was observed in some experiments. The metabolite quantified as 3-OH-BP has been shown to contain a small amount of 9-hydroxybenzo(a)pyrene, and it may also contain other monohydroxybenzo(a)pyrenes (16). The radioactivity was determined by liquid scintillation counting using a Packard Tri-Carb Model 3375 scintillation spectrometer.

**Tracheal Cultures.** The tracheas of both sham- and cigarette smoke-exposed animals were maintained in short-term organ culture for 20 to 24 hr in 10 ml of Leibovitz L-15 medium with 2 mM L-glutamine, 10% fetal calf serum, 100 units penicillin and 100 μg streptomycin per ml, and 0.5 μM [3H]BP as described previously (6). The medium at the end of the culture was extracted with 2 × 10 ml of ethyl acetate, and the BP metabolites were quantified by cutting the TLC plate into 5-mm segments and counting the radioactivity. The total diol fraction including significantly greater radioactivity than that in controls was observed in some experiments. The metabolite quantified as 3-OH-BP has been shown to contain a small amount of 9-hydroxybenzo(a)pyrene, and it may also contain other monohydroxybenzo(a)pyrenes (16). The radioactivity was determined by liquid scintillation counting using a Packard Tri-Carb Model 3375 scintillation spectrometer.

**Covalent Binding.** The covalently bound radioactivity in lungs after perfusion and tracheas after culture was determined essentially by the method of Siekevitz (22). Appropriate tissue blanks were incubated with [3H]BP at 4°C.

**RESULTS**

**Metabolism of Intratracheally Instilled BP (2 nmoles) by Isolated Perfused Lungs of Sham- and Cigarette Smoke-exposed Rats**

BP was metabolized by isolated perfused lungs from both sham- and cigarette smoke-exposed rats to ethyl acetate-soluble metabolites that cochromatographed with 9,10-dihydrodiol, 7,8-dihydrodiol, 4,5-dihydrodiol, and 3-OH-BP (Table 1). Significant amounts of radioactivity migrating near the origin were also detected, particularly in extracts of lungs from the cigarette smoke-exposed animals. Cigarette smoke exposure significantly increased the percentage production of all metabolites and caused a decrease in the percentage of unchanged BP (Table 1).

The perfusate of sham- and cigarette smoke-exposed perfused lungs also contained ethyl acetate-soluble metabolites that cochromatographed with 9,10-dihydrodiol, 7,8-dihydrodiol, and 3-OH-BP (Chart 1). Small amounts of a material (Y) migrating between 4,5-dihydrodiol and 3-OH-BP were observed, particularly in the radioactive profile of the ethyl acetate extract of the perfusate from cigarette smoke-exposed animals (Chart 1b). The percentages of dihydrodiol and phenol metabolites in the perfusates from cigarette smoke-exposed animals were significantly greater than those from the corresponding controls (Table 2). Although the percentage of ethyl acetate metabolites in the perfusate collected from 4 to 8 min appeared to be higher in many cases than that collected during the first 4 min of perfusion (Table 2), little or no differences were observed in the absolute amounts of metabolites in these 2

<table>
<thead>
<tr>
<th>Metabolite(s) of BP</th>
<th>Sham</th>
<th>Smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td>Near origin</td>
<td>0.43 ± 0.11* (3)*</td>
<td>1.10 ± 0.20 (4)*</td>
</tr>
<tr>
<td>9,10-Dihydrodiol</td>
<td>0.26 ± 0.03 (3)</td>
<td>0.98 ± 0.14 (4)*</td>
</tr>
<tr>
<td>7,8-Dihydrodiol</td>
<td>0.03 ± 0.04 (3)</td>
<td>0.98 ± 0.29 (4)</td>
</tr>
<tr>
<td>4,5-Dihydrodiol</td>
<td>0.043 ± 0.019 (3)</td>
<td>0.37 ± 0.09 (4)*</td>
</tr>
<tr>
<td>Total dihydrodiols</td>
<td>0.46 ± 0.13 (3)</td>
<td>2.69 ± 0.54 (4)*</td>
</tr>
<tr>
<td>3-OH-BP</td>
<td>0.99 ± 0.15 (3)</td>
<td>5.48 ± 0.81 (4)*</td>
</tr>
<tr>
<td>BP (unmetabolized)</td>
<td>95.4 ± 0.5 (3)</td>
<td>87.2 ± 0.7 (4)*</td>
</tr>
</tbody>
</table>

* All results are expressed as mean ± S.E. or as the average of 2 determinations.
* Numbers in parentheses, number of experiments.
* p < 0.05 that results are different from those for corresponding sham-exposed animals by Student's t-test.
* p < 0.01 that results are different from those for corresponding sham-exposed animals by Student's t test.
Chart 1. Ethyl acetate-soluble metabolites from the nonrecirculating perfusate (4 to 8 min) of isolated perfused lungs of (a) sham-exposed and (b) cigarette smoke-exposed rats after intratracheal administration of [3H]BP (2 nmoles). The rats were either sham-exposed or exposed to the smoke of 5 cigarettes during 1 hr, 20 hr before sacrifice. The perfusate from 4 to 8 min was extracted with 2 x 1 volume ethyl acetate. The ethyl acetate-soluble metabolites were concentrated and separated by TLC in a mixture of benzene:ethanol (9:1, v/v). S.F., solvent front.

Exposure of animals to cigarette smoke for either 1 or 10 days caused a significant increase in the overall production of all ethyl acetate-soluble metabolites in the lung and the perfusate. The increase in metabolite production varied from 5- to 6-fold depending on the metabolite (Table 3).

Metabolism of Intratracheally Administered BP by Isolated Perfused Lungs of 3-MC-treated Rats

Ethyl acetate-soluble metabolites that cochromatographed with 9,10-dihydriodiol, 7,8-dihydriodiol, 4,5-dihydriodiol, and 3-OH-BP were detected in the lungs and perfusate after intratracheal instillation of [3H]BP (2 nmoles) to lungs of rats pretreated with 3-MC (Table 4). A metabolite
BP Metabolism in Perfused Lungs

Table 2

The effect of cigarette smoking on the percentage of ethyl acetate-soluble metabolites of [3H]BP in the perfusate after intratracheal instillation of [3H]BP to isolated perfused lungs

Rats were either sham-exposed or exposed to the smoke of 5 cigarettes during 1 hr, for either 1 or 10 days. The animals were sacrificed 20 hr after the last smoke exposure, and the lungs were perfused at a flow rate of 10 ml/min for 8 min with 4.5% bovine serum albumin in Tyrode's solution with glucose (22 mw) after the intratracheal instillation of 2 nmols of [3H]BP. The different-timed fractions of perfusate were extracted with 2 x 1 volume of ethyl acetate. The ethyl acetate-soluble metabolites and unchanged BP were concentrated and separated by TLC in a mixture of benzene:ethanol (9:1, v/v). The metabolites were quantified as explained in the legend to Table 1.

<table>
<thead>
<tr>
<th>Metabolite(s) formed or BP unmetabolized in perfusate as % of ethyl acetate-soluble radioactivity</th>
<th>1 day</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>Sham</td>
<td>Smoke</td>
</tr>
<tr>
<td>Near origin</td>
<td>0-4</td>
<td>0.24 ± 0.18* (3)*</td>
</tr>
<tr>
<td>9,10-Dihydrodiol</td>
<td>4-8</td>
<td>0.26 ± 0.08 (3)</td>
</tr>
<tr>
<td>7,8-Dihydrodiol</td>
<td>0-4</td>
<td>0.58 ± 0.08 (3)</td>
</tr>
<tr>
<td>4,5-Dihydrodiol</td>
<td>4-8</td>
<td>0.91 ± 0.10 (3)</td>
</tr>
<tr>
<td>3-OH-BP</td>
<td>0-4</td>
<td>0.50 ± 0.10 (3)</td>
</tr>
<tr>
<td>BP (unmetabolized)</td>
<td>4-8</td>
<td>0.15 ± 0.11 (3)</td>
</tr>
</tbody>
</table>

° Mean ± S.E.
* Numbers in parentheses, number of experiments.
* p < 0.05 that results are different from those of corresponding sham-exposed animals by Student's t test.
* p < 0.01 that results are different from those of corresponding sham-exposed animals by Student's t test.

Table 3

The effect of cigarette smoking on the total amount of ethyl acetate-soluble metabolites formed both in the lung and the perfusate after intratracheal instillation of [3H]BP (2 nmols)

Details are as given in the legends to Tables 1 and 2.

<table>
<thead>
<tr>
<th>Metabolite(s) of BP</th>
<th>1 day</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>Sham</td>
<td>Smoke</td>
</tr>
<tr>
<td>Near origin</td>
<td>0-4</td>
<td>3.05 ± 0.82* (3)*</td>
</tr>
<tr>
<td>9,10-Dihydrodiol</td>
<td>3.58 ± 0.73 (3)</td>
<td>19.9 ± 2.9 (4)*</td>
</tr>
<tr>
<td>7,8-Dihydrodiol</td>
<td>2.46 ± 0.61 (3)</td>
<td>12.6 ± 2.9 (4)*</td>
</tr>
<tr>
<td>4,5-Dihydrodiol</td>
<td>1.28 ± 0.21 (3)</td>
<td>6.89 ± 1.16 (4)*</td>
</tr>
<tr>
<td>Total dihydrodiols</td>
<td>9.17 ± 1.78 (3)</td>
<td>43.3 ± 7.3 (4)*</td>
</tr>
<tr>
<td>3-OH-BP</td>
<td>8.96 ± 1.97 (3)</td>
<td>41.8 ± 4.3 (4)*</td>
</tr>
<tr>
<td>BP (unmetabolized)</td>
<td>848 ± 79 (3)</td>
<td>808 ± 85 (4)</td>
</tr>
</tbody>
</table>

° Mean ± S.E.
* Numbers in parentheses, number of experiments.
* p < 0.05 that results are different from those of corresponding sham-exposed animals by Student's t test.
* p < 0.01 that results are different from those of corresponding sham-exposed animals by Student's t test.

(Y), associated with radioactivity, migrating between 4,5-dihydrodiol and 3-OH-BP, was also observed in the radioactivic profile of the ethyl acetate extract of the perfusate. Inadequate separation of the phenols and quinones after TLC of the ethyl acetate extract from lung of 3-MC-treated animals prevented the quantitation of these metabolites (Table 4). The formation of all metabolites was increased by 3-MC pretreatment compared to that in sham-exposed animals (Table 4). Previous work had shown that the corn oil used to dissolve the 3-MC had no effect on pulmonary metabolism (28).

Metabolism of [3H]BP by Short-Term Tracheal Organ Culture of Sham and Cigarette Smoke-exposed Animals

Tracheas from both sham- and cigarette smoke-exposed animals metabolized BP to ethyl acetate-extractable metabolites that cochromatographed with 9,10-dihydrodiol, 7,8-dihydrodiol, 4,5-dihydrodiol, and 3-OH-BP (Chart 2). No significant differences were observed in formation of the different metabolites between the tracheas of sham- and cigarette smoke-exposed animals.
Radioactivity Covalently Bound to Isolated Perfused Lungs and Tracheas of Sham- and Cigarette Smoke-exposed Rats

Isolated Perfused Lungs. After lungs were perfused with \(^{3}H\)BP (2 nmoles) for 8 min, the lungs and tracheae were removed separately, and the covalently bound radioactivity was determined as previously described. Cigarette smoke exposure for either 1 or 10 days or 3-MC pretreatment significantly increased the amount of covalently bound radioactivity in lung tissue when compared to the corresponding sham-exposed rats (Table 5). The amount of covalently bound radioactivity in the lung of 4°tissue blanks was always significantly lower than experimental values, e.g., a mean value of 6.2 pmoles bound/g lung was obtained for 10-day-exposed animals. However, due to some variability in tissue blanks the results in Table 5 have been presented without subtracting the 4°tissue blank values. No significant differences were observed in the covalently bound radioactivity in the tracheae of sham- and smoke-exposed animals in these perfusion experiments.

Tracheae. No significant differences were observed in the amounts of covalently bound radioactivity after short-term organ culture of \(^{3}H\)BP (0.5 \(\mu\)M) with tracheae of either 1- or 10-day sham- and smoke-exposed animals.

DISCUSSION

Isolated perfused lungs from sham- and cigarette smoke-exposed rats metabolized BP to ethyl acetate-soluble metabolites that cochromatographed with 9,10-dihydrodiol, 7,8-dihydrodiol, 4,5-dihydrodiol, and 3-OH-BP (Table 1). In agreement with our previous observations (6, 28), 3-OH-BP was the major ethyl acetate-soluble metabolite both in the lung (Table 1) and released into the perfusate during the perfusion (Table 2). Exposure to cigarette smoke caused an approximately 5-fold increase in the total formation of all the major metabolites of BP (Table 3). These results are in agreement with several other studies, using lung homogenates and microsomes (1, 2, 13, 21, 26, 30, 32), which showed an increase in 3-OH-BP after exposure to cigarette smoke. Of interest was the increase in dihydrodiol formation after exposure to either cigarette smoke or 3-MC. In perfusion of lungs from sham- or cigarette smoke-exposed or 3-MC-pretreated rats, 9,10-dihydrodiol was the major dihydrodiol formed, followed by 7,8-dihydrodiol and 4,5-dihydrodiol (Tables 3 and 4). Dihydrodiols may be further metabolized to diol-epoxides, e.g., 7,8-dihydrodiol is converted to 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide (25). This diol-epoxide is suspected as being the ultimate carcinogen derived from BP, on the basis of its exceptional mutagenicity (14, 18, 20, 33) and its identification as the metabolite bound to DNA in mouse skin (8, 10), human bronchial mucosa (10), and cultured cells (25), when these systems are exposed to BP.

Exposure to cigarette smoke and 3-MC also caused a significant increase in radioactivity migrating just beyond the origin (Chart 1; Tables 1 to 4). Part of this radioactivity has been identified in a previous study as benzo(a)pyren-3-yl-hydrogen sulfate (5), while the remainder probably represents a combination of several further metabolites of the initial metabolites. An unidentified metabolite (Y) migrating between 4,5-dihydrodiol and 3-OH-BP (Chart 1) was also observed in agreement with our previous study (6).

In parallel studies with lung microsomes, the production of Y and 4,5-dihydrodiol was significantly increased by exposure to cigarette smoke and 3-MC (27). Another indication of the increased pulmonary metabolism of BP in cigarette smoke-exposed and 3-MC-pretreated animals was the increase in the percentage of water-soluble radioactivity (i.e., the radioactivity remaining after extraction with ethyl acetate) in the perfusate when compared to the corresponding sham-exposed animals (unpublished observation). It is difficult to compare the 1- and 10-day experiments because of the changes in basal activity of sham-exposed animals (Table 3). Although many of these changes were not statistically significant, there was a definite trend toward higher activity in the 10-day sham-exposed animals. Thus, when a comparison was made between 1- and 10-day smoke-exposed animals, many of the differences were statistically significant, but some of these changes may have been due to changes in basal levels (Table 3). The increase in covalently bound radioactivity in the lung of 4°tissue blanks was significantly increased by exposure to cigarette smoke for either 1 or 10 days or 3-MC pretreatment.
above results were compared by calculating the percentage increase of metabolite production in smoke-exposed relative to sham-exposed animals, very little difference was seen between 1- and 10-day smoke-exposed animals. Changes in the sham values may have been due to many factors in the environment such as diet, housing, inducing agents, and stress factors involved in repeated handling. Wattenberg (31) has shown that the basal level of AHH activity in lungs of rats fed purified diets is almost zero, and feeding the animals standard diet increases the activity. Thus exposure of the animals to many factors in the environment may have changed the activities of the sham-exposed animals.

Pretreatment of animals with 3-MC caused changes in the metabolite pattern and in the overall production of metabolites (Table 4) which were very similar to cigarette smoke exposure (Chart 1; Table 3). This might lend some support for the PAH nature of the potent inducer of BP metabolism in cigarette smoke. Cigarette smoke condensate (1 mg) contains about 6 ng of PAH of which about 1 ng is BP (34). Okamoto et al. (21) have shown that 1 mg of tobacco condensate gave an induction of AHH activity approximately equivalent to 1 μg of BP. Thus the increase in activity of metabolite production in these and other studies may be due to PAH or to other inducing agents that result in a metabolite pattern similar to that for 3-MC. The increase may also be due to the presence of other agents in cigarette smoke that markedly enhance the ability of the PAH in the condensate to increase metabolite production.

The tracheas of both sham- and cigarette smoke-exposed
of cigarette smoke-exposed animals may be related to the increase in metabolism. Similarly, when no alterations in metabolism were observed, e.g., in the tracheas of sham-exposed and smoke-exposed animals, then no changes in covalently bound radioactivity were observed. Although it is possible that the increase in covalently bound radioactivity in lungs of cigarette smoke-exposed animals may be related to the chemical induction of lung cancer, the nature and the significance of this covalently bound radioactivity still remain to be determined.

Table 5

The covalently bound radioactivity in the lungs after intratracheal instillation of [3H]BP into isolated perfused lungs of sham- and smoke-exposed and 3-MC-pretreated rats

Rats were either pretreated for 3 days with 3-MC (20 mg/kg in corn oil) or sham-exposed or exposed to the smoke of 5 cigarettes during 1 hr for either 1 or 10 days. The animals were sacrificed 20 hr after the last treatment, and the lungs were perfused at a flow rate of 10 ml/min for 8 min with 4.5% bovine serum albumin in Tyrode's solution with glucose (22 mm) after the intratracheal instillation of 2 nmoles of [3H]BP. At the end of the perfusion, the lungs were immediately removed, and the covalently bound radioactivity was determined by the method of Siekevitz (22).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmoles bound/g lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-day sham-exposed</td>
<td>7.6 ± 1.5 (5)^a</td>
</tr>
<tr>
<td>1-day smoke-exposed</td>
<td>13.8 ± 2.7 (5)^a</td>
</tr>
<tr>
<td>10-day sham-exposed</td>
<td>10.9 ± 1.7 (4)^a</td>
</tr>
<tr>
<td>10-day smoke-exposed</td>
<td>27.4 ± 4.0 (4)^a</td>
</tr>
<tr>
<td>3-MC-pretreated</td>
<td>34.5 ± 11.5 (3)^a</td>
</tr>
</tbody>
</table>

^a Results are expressed as mean ± S.E.  
^b Numbers in parentheses, number of experiments.  
^c p < 0.05 that results are different from those of corresponding sham-exposed rats by Student's t test. The values of 3-MC-pretreated rats were compared to the values of sham-exposed rats.  
^d p < 0.01 that results are different from those of corresponding sham-exposed rats by Student's t test.

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REFERENCES


Metabolism and Covalent Binding of $[^3H]$Benzo(a)pyrene by Isolated Perfused Lungs and Short-Term Tracheal Organ Culture of Cigarette Smoke-exposed Rats

Gerald M. Cohen, Pekka Uotila, Jaakko Hartiala, et al.


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