Metabolism of Benzo(a)pyrene, N-Nitrosodimethylamine, and N-Nitrosopyrrolidine and Identification of the Major Carcinogen-DNA Adducts Formed in Cultured Human Esophagus

Curtis C. Harris, Herman Autrup, Gary D. Stoner, Benjamin F. Trump, Elizabeth Hillman, Paul W. Schafer, and Alan M. Jeffrey

ABSTRACT

The wide variation in the world-wide incidence of esophageal carcinoma suggests that environmental agents including chemicals cause this cancer. Since the interaction between chemical procarcinogens and human esophagus has not been studied previously, we examined the metabolic fate of benzo(a)pyrene (BP), N-nitrosodimethylamine (DMN), and N-nitrosopyrrolidine in cultured nontumorous esophagus from two patients with and six patients without esophageal carcinoma. Esophageal explants were cultured in a chemically defined medium for 7 days prior to adding [3H]BP (1.5 µM), [14C]DMN (100 µM), or [14C]N-nitrosopyrrolidine (100 µM) for 24 hr. Radioactivity was found bound to both mucosal protein (BP, DMN, and N-nitrosopyrrolidine) and DNA (BP and DMN). The major carcinogen-DNA adducts were: (a) with BP, N2-[10β-(7,8,9,10-tetrahydrobenzo(a)pyrene)]deoxyguanosine; and (b) with DMN, 7-methylguanine, and O6-methylguanine (ratio of O6-methylguanine to 7-methylguanine was 0.3). The interindividual variations among people in binding levels to mucosal DNA were 99-fold for BP and 10-fold for DMN. In the two cases studied, the variation in binding levels among the three major anatomical segments (proximal, mid, and distal) was less than 2-fold. The wide variation in the world-wide incidence of esophageal carcinoma suggests that environmental agents cause this devastating form of cancer (27, 30). In other regions of the world where carcinoma of the esophagus is one of the most common forms of cancer, e.g., Iran and China, the etiological agents have yet to be positively identified (10, 19, 28).

Carcinoma of the esophagus has been produced in experimental animals by approximately 20 different N-nitrosamines (22). Since these chemicals are procarcinogens, it is important to determine whether the esophagus has the ability to activate N-nitrosamines to ultimate carcinogens. However, to our knowledge, the metabolism of chemical carcinogens including N-nitrosamines by the human esophagus has not been investigated previously. We report here data indicating that cultured human esophagus can metabolize both BP and DMN into electrophiles that bind to cellular DNA.

MATERIALS AND METHODS

Chemicals

[3H]BP (24 or 57 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was generally labeled with [3H] and purified to greater than 99% radiochemical purity by column chromatography (4). [14C]DMN (26, 35, or 57 mCi/mmol; New England Nuclear, Boston, Mass.) was purified to greater than 98% radiochemical purity by the method of den Engelse et al. (8). [14C-2,5]NPy (16.2 mCi/mmol; New England Nuclear) was greater than 99% radiochemical purity by the method of den Engelse et al. (8). [14C]NPy (26, 35, or 57 mCi/mmol; New England Nuclear) was purified to greater than 98% radiochemically pure. Dimethyl sulfoxide (Pierce Chemical Co., Rockford, Ill.) was silylation grade and stored under nitrogen. O6-methylguanine was synthesized (7), and 7-methylguanine and 3-methyladenine were purchased from Vega-Fox Biochemicals, Tucson, Ariz.

Specimens and Culture Conditions

Nontumorous human esophageal tissues were obtained at the time of either surgery (HE 182C, 63-year-old male; HE 214, 56-year-old male, both with esophageal carcinoma) or immediate autopsy (26) (HE 192, 20-year-old male; HE 194, 20-year-old male; HE 195, 21-year-old female; HE 197, 58-year-old male; HE 201, 33-year-old male; HE 203, 33-year-old male; HE 215, 22-year-old female), immersed in L-15 tissue culture medium and stored under nitrogen. O6-methylguanine was synthesized (7), and 7-methylguanine and 3-methyladenine were purchased from Vega-Fox Biochemicals, Tucson, Ariz.

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[3] The abbreviations used are: BP, benzo(a)pyrene; DMN, N-nitrosodimethylamine; NPy, N-nitrosopyrrolidine; HPLC, high-pressure liquid chromatography; BPDE, 1, 2- (7, 8, 9, 10-tetrahydrobenzo(a)pyrene).
were included as UV markers (18). Chromatography before separation by HPLC. The isolated peaks were then chromatographed on a Dupont 830 high-pressure liquid Chromatograph fitted with a Bondapak C-18 column (300 x 3.9 mm; Waters Associates, Inc., Milford, Mass.) using a 1%, Number 2 concave gradient of 30 to 60% methanol in water, and 1-min fractions were collected. Known adducts were included as UV markers (18).

High-Pressure Liquid Chromatographic Analysis of Organic Solvent

Media were extracted 3 times with ethylacetate-acetone (2/1), dried over MgSO4, and evaporated to dryness in vacuo. The residue was dissolved in methanol, and unmetabolized BP was removed by thin-layer chromatography. The metabolites were separated by HPLC using a Zorbax octadecyl silane column (250 x 6.2 mm; DuPont Instruments, Wilmington, Del.). The BP metabolites were eluted using a gradient of 60% methanol in water to 100% methanol with a rate change of 2%/min (31). Fractions of 0.2 ml were collected every min for 100 min, and the radioactivity in each fraction was determined using Aquasol (New England Nuclear) as scintillation cocktail. Authentic BP metabolites were included as standards and monitored by their absorption at 248 nm.

Analysis of Water-soluble Metabolites of BP

The water-soluble metabolites of BP were separated according to the method of Autrup (1). Ice-cold ethanol (15 ml) was added to 5 ml of the medium from cultures that had been incubated with [3H]BP. The solution was kept at 4° for 30 min and then centrifuged at 9000 x g for 15 min to remove any protein precipitate. The supernatant was evaporated to dryness in a vacuum at ambient temperature. The residue was redissolved in 2 ml of 70% ethanol and applied to an alumina column (150 x 15 mm; neutral; Brockmann Activity 1, 80 to 200 mesh) washed previously with 25% formic acid, water, and finally, ethanol. The column was eluted with 100 ml of absolute ethanol at a flow rate of 2 ml/min to remove BP and nonconjugated metabolites and then with 100 ml of water to elute BP-sulfate esters, followed by 100 ml of 0.05 M ammonium phosphate buffer (pH 3) to elute BP-glucoronides, and finally with 25% formic acid to elute BP-glutathione and the more polar conjugates. Aliquots of 200 μl from each of the fractions were assayed for radioactivity by liquid scintillation methods.

Formation of [14C]CO2

In one case (HE 215), the formation of [14C]CO2 from [14C]-DMN was measured as described previously (13). Five explants in 3 sterile, 60-mm plastic Petri dishes (Falcon Plastics, Ox- nard, Calif.) were placed on a rack in a closed container (500-ml Nalgene plastic jar; A. H. Thomas, Philadelphia, Pa.) which was modified with 2 ports for replacing air with 50% O2, 45% N2, and 5% CO2. The containers were flushed with N2, and 5% CO2. The containers were placed on a rocker platform and rocked at approximately 10 cycles/min for 24 hr. To remove 14CO2 formed by the metabolism of the N-nitrosamine, the containers were flushed with N2 for 5 min, and the CO2 was absorbed in 2 serially connected tubes each containing 8 ml of 0.2 M Ba(OH)2. After removal of the explants, 1 ml of 3 M phosphoric acid (pH 3) was added to each culture dish to release CO2 dissolved in the medium. After 4 hr at 37°, the containers were flushed with N2 for another 5 min. The precipitate BaCO3 was collected on Whatman GF/C filters and washed with absolute ethanol until the count in the washing solution was negligible. Medium without explants served as control. The precipitate and filter were suspended in 3 ml of water and 10 ml of Aquasol liquid scintillation cocktail to make a gel (New England Nuclear) and counted.

RESULTS

Data indicating metabolism of BP into water-soluble metabolites and organic solvent-extractable metabolites are shown in Tables 1 to 3. The metabolism of BP into water-soluble
Metabolism of Chemical Carcinogens by Human Esophagus

Table 1
Metabolism of BP by cultured human esophagus

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anatomical segment</th>
<th>Total metabolism</th>
<th>Water-soluble metab (as % of total metab)</th>
<th>Glucuronide conjugates</th>
<th>Glutathione conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE 182C</td>
<td>Distal</td>
<td>145</td>
<td>54</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>HE 192</td>
<td>Middle</td>
<td>51</td>
<td>6</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>HE 195</td>
<td>Proximal</td>
<td>56</td>
<td>1</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>HE 197</td>
<td>Distal</td>
<td>32</td>
<td>2</td>
<td>42</td>
<td>13</td>
</tr>
<tr>
<td>HE 203</td>
<td>Middle and distal</td>
<td>27</td>
<td>16</td>
<td>53</td>
<td>10</td>
</tr>
<tr>
<td>HE 214</td>
<td>Distal</td>
<td>19</td>
<td>15</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>HE 215</td>
<td>Proximal</td>
<td>37</td>
<td>11</td>
<td>35</td>
<td>20</td>
</tr>
</tbody>
</table>

* nmol/100 μg DNA.

Table 2
Pattern of organic-solvent-extractable metabolites of BP formed by cultured human esophagus

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time (min)</th>
<th>HE 182C</th>
<th>HE 192</th>
<th>HE 194</th>
<th>HE 197</th>
<th>HE 214</th>
<th>HE 215</th>
</tr>
</thead>
<tbody>
<tr>
<td>(7,10,9)-Tetrol</td>
<td>10.0</td>
<td>ND</td>
<td>ND</td>
<td>3.9</td>
<td>3.2</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>(7,9,10)-Tetrol</td>
<td>13.2</td>
<td>2.6</td>
<td>2.2</td>
<td>2.5</td>
<td>4.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(7,9,10,9)-Tetrol</td>
<td>16.4</td>
<td>3.6</td>
<td>4.3</td>
<td>3.8</td>
<td>4.1</td>
<td>10.0</td>
<td>9.6</td>
</tr>
<tr>
<td>(7,9,10)-Tetrol; (7,9,10)-triol; and trans-8,10-diol</td>
<td>19.8</td>
<td>13.7</td>
<td>7.2</td>
<td>11.1</td>
<td>14.5</td>
<td>26.3</td>
<td>31.5</td>
</tr>
<tr>
<td>trans-4,5-Diol</td>
<td>28.9</td>
<td>2.3</td>
<td>2.1</td>
<td>6.9</td>
<td>2.1</td>
<td>11.2</td>
<td>4.3</td>
</tr>
<tr>
<td>trans-7,8-Diol</td>
<td>31.5</td>
<td>6.1</td>
<td>7.4</td>
<td>8.8</td>
<td>3.0</td>
<td>12.9</td>
<td>10.6</td>
</tr>
<tr>
<td>3-Hydroxy</td>
<td>44.1</td>
<td>3.2</td>
<td>5.3</td>
<td>6.8</td>
<td>5.8</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>9-Hydroxy</td>
<td>45.8</td>
<td>4.3</td>
<td>12.7</td>
<td>5.2</td>
<td>9.7</td>
<td>2.5</td>
<td>6.4</td>
</tr>
<tr>
<td>1,6-Quinone</td>
<td>47.4</td>
<td>13.7</td>
<td>8.7</td>
<td>4.4</td>
<td>7.8</td>
<td>3.9</td>
<td>7.2</td>
</tr>
<tr>
<td>3,6-Quinone</td>
<td>50.0</td>
<td>16.5</td>
<td>11.7</td>
<td>11.7</td>
<td>9.1</td>
<td>8.7</td>
<td>10.0</td>
</tr>
<tr>
<td>6,12-Quinone</td>
<td>61.0</td>
<td>11.0</td>
<td>15.8</td>
<td>19.2</td>
<td>17.1</td>
<td>5.2</td>
<td>3.0</td>
</tr>
<tr>
<td>Unidentified</td>
<td>23.0</td>
<td>22.6</td>
<td>15.7</td>
<td>20.4</td>
<td>12.2</td>
<td>15.7</td>
<td></td>
</tr>
</tbody>
</table>

* (7,10,9)-Tetrol, tetrahydroxytetrahydrobenzo(a)pyrene in which the 10-hydroxy is cis and 8-hydroxy and 9-hydroxy are trans to the reference 7-hydroxy, respectively (other tetrols and triols are designated similarly). trans-9,10-diol, trans-9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (other diols are designated similarly).

metabolites varied among the 8 patients from 1 to 68% of the total metabolism. While the variation among specimens from different donors was wide, i.e., 68-fold, the variation found within a single case, including a comparison of data from the different anatomical segments of the esophagus, was narrow, i.e., 2-fold. In spite of the wide interindividual variation in the total formation of water-soluble metabolites, the percentages of sulfate esters (range, 21 to 55%), glucuronide conjugates (7 to 37%), and glutathione conjugates (24 to 66%) formed with BP metabolites from each of the cases studied indicated that the patterns of metabolites were qualitatively similar in all cases.

Most of the radioactivity of organic solvent-extractable metabolites of BP released into the medium by the cultured esophageal explants cochromatographed with authentic metabolites of BP, including the proximate carcinogenic metabolite of BP, (−)-trans-7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (Tables 2 and 3). When the 3 major anatomical segments of the esophagus were compared, the patterns of BP metabolites were similar.

Binding levels of [3H]BP, [14C]DMN, and [14C]NPy to mucosal cell DNA and protein are listed in Tables 4 and 5. Binding of BP to both DNA and protein was detected in the 8 cases studied. In each case, the binding level to protein was greater than to DNA. The interindividual variations were 99-fold (range, 0.3 to 29.9) for binding to DNA and 9-fold (range, 21 to 194) for binding to protein. When the binding levels of BP to DNA were compared among the 3 major anatomical segments, the binding levels were similar in magnitude. Chromatographic analysis of the BP-DNA adducts is shown in Chart 1. The 3 major adducts are formed between BPDE I and guanine. Peaks A and B have not been identified as yet, but they elute in the region of adducts between BPDE I and deoxytocysine.

When either [14C]DMN or [14C]NPy was incubated with explants of esophagus, radioactivity was tightly bound to cellular protein, but only metabolites of DMN were bound to DNA (Tables 4 and 5). Methylation of DNA following incubation of the explants with DMN was detected by separating the methylated bases with Durrum 1-A resin chromatography. Three methylated bases were identified, and the levels of methylation (μmol of methylated base per mol of guanine) were: 06-methylguanine, 76; 3-methyladenine, 58; and 7-methylguanine,

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Table 3
Pattern of organic-solvent-extractable metabolites of BP formed by different anatomical segments of cultured human esophagus

Table 4
Binding of chemical carcinogens to DNA in cultured human esophagus

Table 5
Binding of chemical carcinogens to protein in cultured human esophagus

DISCUSSION

258. The binding levels of DMN to DNA varied widely and ranged from nondetectable (<10) to 1109 pmol/10 mg of DNA. 14CO2 liberated from [14C]DMN was measured in one case (HE 215). The values for the upper, middle, and lower anatomical esophageal segments were 22.4, 15.5, and 11.7 pmol of [14C]CO2 per 10 mg of DNA, respectively.

Positive correlations for carcinogen-binding levels to cellular DNA were found (DMN versus BP, r = 0.89, p < 0.05); i.e., if the binding level of BP was high, then the binding level of DMN was also high in that same case, etc. The correlations for carcinogen-protein adducts were: (a) NPy versus DMN, r = 0.84, p < 0.05; (b) DMN versus BP, r = 0.32, p > 0.05; and (c) NPy versus BP, r = 0.03, p > 0.05.

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and water-soluble metabolites were detected (Tables 1 to 3). In the small number of cases studied to date, large quantitative differences in total metabolism were found. These interindividual variations were not related to detectable differences in metabolism by the 3 major anatomical segments of the esophagus. While a wide variation in quantitative levels was found among individuals, analysis of the patterns of both water-soluble and organic solvent-extractable metabolites revealed only minor qualitative differences. A similar observation has been made in studies with other human tissues and cells including pulmonary macrophages (4), colon (6), bronchus (16), and peripheral lung (25).

A wide interindividual variation in binding levels of BP to DNA was found in studies with various human tissues: human esophagus, 99-fold (8 cases); bronchus, 75-fold (57 cases) (12, 14); and colon, 100-fold (54 cases) (2). We cannot rule out the possibility that subtle and persistent changes in cellular physiology caused by either the culture conditions or environmental factors that altered the in situ esophageal mucosa in the donor prior to culture of the tissue may, in part, be responsible for these quantitative differences. In contrast to the variation in binding levels of BP to DNA, the variation in both binding levels of BP to protein (8-fold; range, 23 to 194) and total metabolism of BP (12-fold; range, 19 to 232; Table 1) was not as wide.

The major BP-DNA adduct formed in the esophageal mucosa was the result of a trans addition of the 2-amino group of guanine to the 10-position of BPDE I (Chart 1). Both stereoisomeric forms of BPDE I form adducts with DNA. However, the 7R form of BPDE I bound to guanine is predominant. Our previous studies have identified these BP-DNA adducts in cultured human esophagus. This radioactivity was not due to contamination from tetrads of BP and may be deoxyguanosine derivatives which elute in this region of the chromatogram (Chart 1, Peaks A and B; Ref. 18).

N-Nitrosamines were also metabolized by cultured human esophagus. DMN was metabolized to CO$_2$ and to carbonium ions; the latter caused methylation of DNA bases and formed 0'-methylguanine, 3-methyladenine, and 7-methylguanine. While 0'-methylguanine could lead to base mispairing during DNA synthesis (11, 21) and thus may be an important factor in mutagenesis and carcinogenesis, the importance of this methylated base in carcinogenesis of the human esophagus remains to be determined.

In contrast to the findings with DMN, an acyclic aliphatic N-nitrosamine, detectable levels of radioactivity from a cyclic N-nitrosamine, [14C]NPy, were found bound only to protein. In each case studied, the binding levels to protein were 2- to 5-fold higher than were those found with DMN. When compared to esophagus, cultured human bronchus and colon can each activate [14C]NPy to metabolites that bind to both DNA and protein (5, 13). Therefore, the lack of detectable levels of radioactivity of [14C]NPy bound to DNA in esophagus may be an example of different metabolic capabilities of different organs in humans.

In conclusion, cultured human esophagus can activate BP and DMN into metabolites that bind to cellular DNA and protein. The major carcinogen-DNA adducts are identical to those found in human bronchus and colon. These carcinogen-DNA adducts and the metabolic pathways leading to them are similar to those found in experimental animals that are susceptible to the carcinogenic action of these environmental chemicals. The biochemical data reported here enhance our confidence in the extrapolation of carcinogenesis data from experimental animals to humans.

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C. C. Harris et al.


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