Benzo(a)pyrene Enhances Atherosclerosis in White Carneau and Show Racer Pigeons

Jane L. Hough, Malcolm B. Baird, George T. Sfeir, Catherine S. Pacini, Diane Darrow, Catherine Wheelock

Benzo(a)pyrene (BaP), a major environmental pollutant and component of cigarette smoke, is both carcinogenic and atherogenic in experimental models. We investigated the effect of long-term administration of BaP on atherosclerosis in both atherosclerosis-susceptible White Carneau (WC) and atherosclerosis-resistant Show Racer (SR) pigeons. The number and size of arterial lesions in the brachiocephalic arteries in WC and SR females but not males were significantly enhanced after long-term dosing with BaP. Metabolic activation appears to be required for BaP atherogenicity, since benzo(e)pyrene (BeP), a noncarcinogenic analogue of BaP, did not enhance lesion development. Studies with 3H-BaP revealed no significant differences between male and female or between WC and SR pigeons in the arterial distribution of BaP and/or its metabolites. There were no consistent differences in blood pressure or plasma cholesterol levels between breeds or sexes. However, chronic administration of BaP did result in complete infertility in female birds, concomitant with grossly visible changes in ovarian appearance. These results clearly show that long-term dosing with BaP alters ovarian structure and function in treated birds, at the same time aggravating the development of arterial lesions. Thus, BaP-induced atherogenicity in female pigeons may be a consequence of an alteration in estrogen production or of antiestrogenic properties of BaP at the level of the arterial wall and may serve as a highly useful animal model to examine the well-known rapid development of atherosclerosis in postmenopausal women. (Arterioscler. Thromb. 1993;13:1721-1727.)

KEY WORDS • atherosclerosis • benzo(a)pyrene • pigeons • arterial change • females

Smooth muscle cell proliferation has long been recognized as an early feature of atherosclerosis. The monoclonal nature of some atherosclerotic plaques and their similarities to smooth muscle tumors of the uterus support the theory that arterial lesions are of neoplastic origin. Thus, it is important to determine those features of atherogenesis and carcinogenesis that are common to both processes, an issue of particular interest when atherosclerosis is aggravated by known carcinogens.

Albert et al. demonstrated that treatment of chickens with various chemical carcinogens resulted in the production of atherosclerotic lesions. These findings stimulated studies in the White Carneau (WC) pigeon, which develops atherosclerotic lesions spontaneously with age, and Revis et al. reported that carcinogenic polycyclic aromatic hydrocarbons (PAHs) aggravated the development of atherosclerosis in male WC pigeons. However, aggravation of lesion formation was not related to carcinogen-induced alterations in blood pressure, plasma cholesterol levels, or lipoprotein profiles.

It has long been known that atherosclerotic lesions develop spontaneously in the arteries of WC pigeons at a much higher incidence than do corresponding lesions in Show Racer (SR) pigeons. Differences between breeds in the susceptibility to the development of atherosclerosis cannot be explained by differences in known risk factors, including plasma cholesterol levels, lipoprotein distribution, or blood pressure. In addition, the SR pigeon maintains its relative resistance to atherosclerosis compared with the WC pigeon even when the former is fed levels of cholesterol that are sufficiently large to cause radical yet similar changes in the physicochemical nature of lipoproteins in both strains of birds. However, as with naturally occurring atherosclerosis, no difference in known risk factors could explain the breed differences in susceptibility to the disease in cholesterol-fed pigeons.

The effect of carcinogens on atherogenesis in the resistant SR pigeon is not known. Prichard et al. injected several breeds of pigeons with the PAH dibenz[a,h]anthracene to determine whether a correlation existed between susceptibility to the formation of atherosclerotic lesions and sarcoma induction. No correlation was found, and there were no significant differences between SR and WC pigeons. The role of this PAH as an atherogen was not specifically investigated, but the results suggested that metabolism of carcinogenic PAH might be similar for both the SR and WC strains.

We have initiated studies to elucidate the mechanism for carcinogen-induced atherogenesis by examining the effects of the carcinogen benzo(a)pyrene (BaP) and benzo(e)pyrene (BeP), a noncarcinogenic analogue, on the development of atherosclerotic lesions in the resistant SR and the susceptible WC pigeon. Female pigeons were included in these studies to discern any differential
effect of carcinogen treatment between the sexes. Unexpectedly, and of potentially great interest, long-term administration of BaP produced a significant increase in lesion size and number in the brachiocephalic arteries of females but not in males. Furthermore, carcinogen-induced aggravation of lesion development in females occurred concomitantly with grossly apparent changes in ovarian appearance and complete loss of fertility. These results suggest that this animal model may be of benefit in understanding the mechanism for the rapid development of atherosclerosis in postmenopausal women, particularly when such development is further aggravated by smoking. A preliminary abstract of some of the present results has appeared elsewhere.10

Methods

**Chemicals**

BaP and BeP were purchased from Aldrich Chemical Co, Milwaukee, Wis, whereas 3H-BaP was purchased from New England Nuclear Research Products, Boston, Mass. These compounds were used without further purification. Corn oil was 100% pure expeller pressed (Hain Pure Food Co, Inc, Los Angeles, Calif) and was kept frozen before use to prevent oxidation over the 5-month experimental period.

**Animals and Experimental Procedure**

WC and SR pigeons were obtained from The Bowman Gray School of Medicine/Research Farm, Winston-Salem, NC. Birds 3 to 6 months of age were maintained at ambient temperature, with 3 or 4 birds per cage. The birds were fed Purina pigeon chow and presented with tap water ad libitum. All animal procedures were in accordance with institutional guidelines.

Pigeons were randomly segregated into three groups and injected once weekly, intramuscularly, with BaP (10 mg/kg body weight, 12.5 mg/mL corn oil), BeP (10 mg/kg body weight, 12.5 mg/mL corn oil), or vehicle (corn oil alone). Birds were weighed weekly, while tissue and plasma samples according to the method of Aldrich Chemical Co.

**Analysis of Arterial Lesions**

At the end of the experimental period the birds were killed, and the aortic tree within the chest wall was dissected free. The brachiocephalic and thoracic arteries were removed, rinsed, and dissected free of adventitia and adhering connective tissue. The arteries were fixed overnight in 60% isopropanol, stained overnight with oil red O (in 60% isopropanol), and destained in 60% isopropanol for 4 hours, with a change of destaining solution once every hour. Lesions were defined as oil red O-positive areas and examined en face with a light microscope. The length and width of each lesion was determined with an ocular micrometer, and area size of each lesion in square millimeters was calculated as the resultant product.

**Hepatic Cytochrome Content, Ethoxyquin, O-Deethylase Activity, and Protein Determination**

Hepatic microsomes were isolated according to the method of Baird et al,11 and cytochrome P450 and cytochrome b5 contents were estimated by difference spectroscopy.12 NADPH cytochrome c reductase activity was determined by the method of Williams and Kamin13 and ethoxyquin O-deethylase according to Birnbaum.14 All results were normalized to protein content in the microsomal sample as determined by the method of Lowry et al,15 using crystalline bovine serum albumin as a standard.

**Plasma Lipoproteins**

Plasma lipoproteins were separated by differential centrifugation. Plasma was spun sequentially for the isolation of very-low-density lipoprotein plus intermediate-density lipoprotein, low-density lipoprotein, and high-density lipoprotein at d<1.019, d<1.063, and d>1.063 g/mL, respectively. All plasma and lipoprotein samples were extracted by partitioning into hexane from 42% ethanol in water according to the method of Thompson et al.16 Aliquots of the hexane phase were used for determination of cholesterol by the method of Zak et al17 after saponification according to Abell et al.18

**Arterial Blood Pressure**

Blood pressure was determined manometrically by direct cannulation of the right alar (wing) artery.

**Tissue Distribution of BaP**

The distribution of BaP in pigeon tissues after administration was determined in untreated birds using tritiated BaP. Radiolabeled tracer plus cold BaP (10 mg/kg, in corn oil) was injected into the pectoral muscle, and blood samples were taken from the right alar vein at 10 minutes and at 1 and 4 hours after injection. The birds were killed 24 hours after injection. Blood was collected, and tissues were harvested and analyzed for the presence of the radiolabel. Lipids were extracted from tissue and plasma samples according to the method of Radin.19 The lipid fraction was back-extracted twice with disodium sulfate to remove polar substances. The resulting lipid fraction was considered to contain only the parent compound, BaP, while the disodium sulfate extracts contained phase 1 metabolites of BaP. The pellet remaining after extraction was then sequentially hydrolyzed to obtain RNA, DNA, and protein. Two milliliters of 0.155 mol/L KCl was added to the pellet and vortexed for 30 seconds. One milliliter of 0.6N perchloric acid (PCA) was added and the sample placed in an ice bath for 15 minutes. The sample was then centrifuged at 2800 rpm for 10 minutes. The supernatant was removed and the pellet washed twice with 3 mL of 0.2N PCA. The washes were combined with the supernatant (acid-soluble fraction containing conjugated BaP metabolites) and saved for radioactivity determination. Four milliliters of 0.3N KOH was then added to the resulting pellet, which was vigorously vortexed for 30 seconds and incubated overnight at 37°C. After overnight incubation the sample was vortexed, 2 mL of ice-cold 1.2N PCA was added, and the sample was reagitated. The sample was then centrifuged at 2800 rpm for 10 minutes. The supernatant (RNA...
fraction) was removed and the pellet washed twice with 2 mL of 0.2N PCA. Four milliliters of 0.5N PCA was gradually added to the pellet, which was then heated to 70°C for 20 minutes. The sample was then centrifuged at 2800 rpm for 10 minutes and the supernatant (DNA fraction) removed and saved. The last digestion was repeated to ensure hydrolysis of all DNA in the pellet.

Distribution of BaP, referred to as influx, was estimated by dividing tissue radioactivity by the area under the plasma radioactivity curve, which was taken from the time of injection to the termination of the experiment, and was expressed as microliters of plasma per gram of tissue per hour. This term expresses influx as fraction removed and saved. The last digestion was repeated to ensure hydrolysis of all DNA in the pellet.

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**Statistical Analysis**

Multifactor ANOVA was used to determine the significance of the effects of treatment, sex, and breed on the development of atherosclerotic lesions. Tukey's procedure was used for pairwise comparison of groups. The Student's t test was used for determination of significance of the uptake and metabolism study.

**Results**

**BaP Enhancement of Atherosclerotic Lesions**

The data for 3- and 5-month treatment periods were pooled, since no differences between the two treatment periods were found for any of the groups. BaP treatment of both WC and SR female birds significantly increased the number and size of atherosclerotic lesions in the brachiocephalic arteries when compared with control and BeP-treated females and males (all treatments; Table 1). Lesion size and number in the brachiocephalic arteries from BaP-treated male birds tended to be slightly higher than control, although enhancement of lesion size and number was not statistically significant. BeP treatment did not have any effect on lesion size and number. There was also no significant effect of breed on lesion size and number in the brachiocephalic arteries.

Unlike in the brachiocephalic arteries, the effect of carcinogen treatment on lesion size in the thoracic artery was not quite significant (from ANOVA, \(P=0.059\)). However, both breed and sex produced significant effects on thoracic lesion size (\(P<0.0001\) and \(P=0.0003\), respectively; Table 1). Lesions of the thoracic aorta from BaP-treated WC females were significantly greater in size than lesions from birds in all other groups except BeP-treated WC females. BaP treatment tended to increase lesion size in the thoracic aorta of SR female pigeons, although this effect was not statistically significant. As in the brachiocephalic arteries, treatment did not significantly affect lesion size in thoracic arteries from male birds. Lesion size in thoracic arteries from SR pigeons as a group were significantly lower than in WC pigeons (\(P<0.05\)).

The number of lesions in the thoracic artery did show a significant effect of treatment in addition to a significant effect of breed and sex. When the breeds were pooled, the BaP-treated females carried a significantly greater (\(P<0.05\)) number of lesions compared with all other groups. WC males and females had a significantly
TABLE 2. Hepatic Microsomal Cytochrome P450 and Cytochrome b5 Contents in Carcinogen-Treated Pigeons

<table>
<thead>
<tr>
<th>Breed</th>
<th>Sex</th>
<th>No. of Birds</th>
<th>Liver Weight, g</th>
<th>Cytochrome P450 Content, nmol/mg Protein</th>
<th>Cytochrome b5 Content, nmol/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Carneau</td>
<td>M</td>
<td>10 Control</td>
<td>6.33±0.40</td>
<td>0.71±0.09</td>
<td>0.44±0.05*</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>11 BeP</td>
<td>6.10±0.32</td>
<td>0.80±0.08#</td>
<td>0.67±0.06†</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10 BaP</td>
<td>7.26±0.32#</td>
<td>0.99±0.09†</td>
<td>0.81±0.08§</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8 Control</td>
<td>7.12±0.35</td>
<td>0.49±0.11</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7 BeP</td>
<td>7.17±0.61</td>
<td>0.35±0.05#</td>
<td>0.30±0.50†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6 BaP</td>
<td>8.82±0.31†</td>
<td>0.49±0.09†</td>
<td>0.41±0.09§</td>
</tr>
<tr>
<td>Show Racer</td>
<td>M</td>
<td>9 Control</td>
<td>6.56±0.52</td>
<td>0.42±0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6 BeP</td>
<td>5.38±0.24</td>
<td>0.83±0.09</td>
<td>0.56±0.06</td>
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<td>0.91±0.07</td>
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<tr>
<td></td>
<td>F</td>
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<td>0.44±0.05</td>
<td>0.40±0.04</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>11 BeP</td>
<td>6.44±0.21</td>
<td>0.52±0.06</td>
<td>0.44±0.04</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7 BaP</td>
<td>7.64±0.67#</td>
<td>0.44±0.10</td>
<td></td>
</tr>
</tbody>
</table>

BeP indicates benzo(e)pyrene and BaP, benzo(a)pyrene. Values are mean±SEM.

*†‡§||Means with the same symbol are significantly different at P<.01.

#**Means with the same symbol are significantly different at P<.05.

#**Pooled BeP-treated pigeons were significantly different from BeP-treated and control pigeons at P<.01.

larger number of lesions than SR males and females (treatments pooled, P<.01).

Analysis of the Hepatic Mixed-Function Oxidase System (MFOS)

Liver weights in BaP-treated birds were significantly higher than in BeP-treated and control birds. Liver weight did not vary significantly between male and female birds, however (Table 2). There were no differences in hepatic microsomal cytochrome P450 and cytochrome b5 content or in 7-ethoxycoumarin O-deethylase activity between pigeon breeds in the control or treatment groups (Table 2). However, BeP- and BaP-treated males showed significant induction of the content of both cytochromes and of 7-ethoxycoumarin O-deethylase activity over those present in control birds and treated females. There were no changes in these parameters in female birds despite the fact that only in females were significant differences seen in lesion size and number. There were no significant differences in cytochrome c reductase activity between any of the groups.

In Vivo Distribution and Metabolism of BaP

To determine whether there was an inherent difference between male and female pigeons in arterial interaction with BaP, we measured the in vivo distribution and metabolism of 3H-BaP by the brachiocephalic and thoracic arteries from untreated male and female WC and SR pigeons. Intramuscular injection of radiolabeled BaP into the pectoral muscles of WC and SR pigeons resulted in the rapid appearance of significant levels of BaP in the blood (within 10 minutes). BaP is rapidly removed from the blood by all major organs. The tissues then metabolize, conjugate, and secrete BaP into the blood for eventual excretion in the urine. Thus, the amount of BaP radiolabel in the blood represents metabolites rather than the parent compound and increases rapidly with time, and by 24 hours BaP is present in the blood mainly as metabolites (data not shown). These metabolites actually appear to accumulate over a 24-hour period and are not as readily removed from the circulation as the original compound.

As early as 6 hours after injection, only 4% to 5% of the BaP label in the liver is present as the parent compound. Forty percent occurs as phase I metabolites, with the remaining label bound to RNA, DNA, protein, and sugars. At 24 hours 2% to 4% of the label present is the parent compound, while 40% to 80% represents phase II metabolites in the liver and >80% in the heart. However, 10% to 30% of the label present in the artery 24 hours after injection is present as the parent compound, reflecting a lower capacity for metabolism (activation) of BaP by arterial tissue. The slow rate of metabolism of BaP in arteries may actually result in prolonged exposure of the tissue to a higher level of activated compounds (carcinogenic metabolites) than is the case in tissues that metabolize BaP more rapidly.

There were no significant differences in the distribution of BaP in the arteries, livers, or hearts from male and female WC and SR pigeons 24 hours after administration of the carcinogen (Table 3). There were also no significant differences in the amount of radiolabeled BaP bound to macromolecules (Table 4). BaP in tissues was higher when determined at shorter time periods, a consequence of metabolism of the parent compound and its release back into the bloodstream. Thus, the proportion of radiolabel in the blood representing the parent compound is greatly diminished with time, and the area under the entire radioactivity curve for the 24-hour experimental period greatly overestimates the amount of the parent compound that is available to tissues for influx and metabolism. However, calculation of the tissue distribution of the radiolabel using the area of the curve for the parent compound likewise showed no effect of breed or sex on tissue distribution of the radiolabel.

Cholesterol and Blood Pressure Analysis

Both BaP and BeP treatment increased plasma cholesterol levels (data not shown). This increase was
partially marked in some of the females. However, no correlation was found between lesion size, the sum of the total area of thoracic and brachiocephalic lesions in individual birds, and plasma cholesterol levels (Figure). There were also no consistent differences in lipoprotein profiles. Blood pressure was measured on selected birds 1 to 3 days before they were killed. No significant differences in blood pressure were found between breed, sex, or treatment groups (data not shown).

Discussion
Revis et al.5 have reported that male WC pigeons showed a significant enhancement of aortic atherosclerosis with BaP treatment after 3 and 6 months. However, the present study (3 and 5 months of treatment) revealed that BaP dosing significantly increased lesion size and number in the brachiocephalic arteries in female birds but not in males. Thoracic lesion size and number as reported by Revis et al.5 for BaP-treated male birds were similar to those reported here. However, the lesion size and number for the control birds were higher in the present study than in previous studies.5 The development of atherosclerosis is known to be affected by maintenance of the birds in cages (as in the present study) as opposed to in fly coops, in which the birds are able to form pairs.20 Revis et al.5 did not report how the birds in their study were maintained.

The present results show that female birds had lesions in the brachiocephalic arteries that were much larger than those reported by Revis et al.5 Thus, the results of both studies show that female birds are much more sensitive to BaP treatment than are male birds. Male pigeons did show a tendency for increased lesion size and number with BaP treatment. However, a longer treatment period is required to determine whether BaP exerts a significant effect on atherogenesis in males. Finally, the present study also confirms the results of Revis et al.,5 in that the lesion enhancement after BaP treatment cannot be explained by changes in plasma cholesterol levels, lipoprotein profiles, or blood pressure.

BaP is a procarcinogen and must generally be metabolized to reactive electrophiles by the microsomal MFOS to exhibit carcinogenicity. The results of this investigation and that of Revis et al.5 indicate that metabolism of BaP is also necessary for it to exhibit

### Table 3

<table>
<thead>
<tr>
<th>Breed</th>
<th>Sex</th>
<th>Time, h</th>
<th>Thoracic</th>
<th>Brachiocephalic</th>
<th>Liver</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td>F</td>
<td>6</td>
<td>11.0±6.00</td>
<td>1.06±0.09</td>
<td>0.21±0.08</td>
<td>0.17±0.17</td>
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<td>M</td>
<td>12</td>
<td>21.8±0.20</td>
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<td>F</td>
<td>24</td>
<td>0.12±0.06</td>
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<td>F</td>
<td>24</td>
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<td>0.13±0.05</td>
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<td>1.54±0.37</td>
</tr>
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</table>

*The amount of benzo(a)pyrene bound to macromolecular components is expressed as a percent of the total benzo(a)pyrene in the tissue 24 hours after injection.

### Table 4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Breed</th>
<th>Sex</th>
<th>Thoracic</th>
<th>Brachiocephalic</th>
<th>Liver</th>
<th>Heart</th>
</tr>
</thead>
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<td>DNA</td>
<td>WC</td>
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<td>3.1±2.3</td>
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<td>5.3±0.4</td>
<td>1.2±0.1</td>
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<tr>
<td></td>
<td>WC</td>
<td>M</td>
<td>9.7±4.5</td>
<td>6.7±4.3</td>
<td>6.9±1.8</td>
<td>1.3±0.2</td>
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<tr>
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<td>F</td>
<td>10.5±5.7</td>
<td>8.2±4.2</td>
<td>7.6±0.9</td>
<td>1.2±0.1</td>
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<tr>
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<td>SR</td>
<td>M</td>
<td>12.9±4.9</td>
<td>7.0±4.0</td>
<td>5.7±1.2</td>
<td>1.4±0.1</td>
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<tr>
<td>RNA</td>
<td>WC</td>
<td>F</td>
<td>16.5±7.2</td>
<td>10.6±4.0</td>
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<td>5.9±0.7</td>
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<tr>
<td></td>
<td>WC</td>
<td>M</td>
<td>16.0±4.9</td>
<td>10.7±3.7</td>
<td>17.6±7.5</td>
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<td>F</td>
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<td>18.1±6.2</td>
<td>27.5±11.4</td>
<td>3.7±1.2</td>
<td>4.0±0.6</td>
</tr>
</tbody>
</table>

*The amount of benzo(a)pyrene bound to macromolecular components is expressed as a percent of the total benzo(a)pyrene in the tissue 24 hours after injection.
atherogenicity, since BeP, which is not readily metabolized by the MFOS and is considered to be only weakly carcinogenic, if at all, does not enhance atherogenesis in SR and WC pigeons. Majesky et al.21 have measured the biotransformation of BaP by microsomes isolated from the livers and arteries of WC and SR pigeons. They reported a significantly higher percent induction of enzyme activities in the WC than the SR pigeon after administration of 3-methylcholanthrene. However, the actual enzyme activities were not significantly different. In fact, the basal levels of enzyme activities were higher in the SR than the WC birds. We have used the Ames Salmonella mutagenicity test22 to determine the relative capability of microsomes isolated from the liver, lung, and kidneys of WC and SR pigeons to activate BaP. We found no differences in BaP activation by microsomes from 3- to 4-month- or 5- to 6-year-old normal and Aroclor 1254-induced WC and SR pigeons (J.L. Hough, PhD, et al, unpublished data). The results of the present investigation also did not show any differences between WC and SR pigeons in hepatic MFO content and activity. It is of interest that only BaP- and BeP-treated male birds exhibited induction of hepatic microsomal cytochrome P450 and of ethoxycoumarin O-deethylase activity, despite the fact that only in females pigeons, can competitively inhibit estrogen binding receptors or effect a decrease in the nuclear estrogen receptor, since noncarcinogenic PAHs do not possess antithrombin properties.41

Thus, long-term administration of BaP to female pigeons may result in a real decrease in the concentration of estrogen that arrives at the arterial wall or in a functionally apparent decrease in estrogen levels through competitive inhibition of estrogen binding, carcinogen-induced decrease in receptor density, or both. Regardless of the actual mechanism, the BaP-treated female pigeon appears to be an extremely useful model for understanding the mechanism for the well-known rapid development of atherosclerosis in estrogen-deficient postmenopausal women, particularly in those instances when atherogenesis may be also exacerbated by cigarette smoking.

Although no sex difference has been reported in the development of the naturally occurring or cholesterol-aggravated disease in WC pigeons,67 the development of lesions in males and females may result from the interaction of different factors. The administration of BaP alters or provides another factor in females compared with males. Studies have been initiated to determine whether a longer treatment period might result in significant enhancement of lesion development in BaP-treated males and to determine how BaP treatment alters reproductive function, hormone homeostasis, and arterial influx and metabolism in female birds. It is of particular interest that there were no significant differences between WC and SR pigeons that were attributable to BaP treatment. Therefore, whatever the mechanism of BaP enhancement of atherosclerosis, the present study shows that resistance to naturally occurring and cholesterol-aggravated atherosclerosis does not confer resistance to carcinogen-aggravated atherosclerosis and that atherogenesis in pigeons, as in humans, is multifactorial.

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