Effects of Subchronic Exposures to Concentrated Ambient Particles (CAPs) in Mice: V. CAPs Exacerbate Aortic Plaque Development in Hyperlipidemic Mice

Lung Chi Chen and Christine Nadziejko

Nelson Institute of Environmental Medicine, Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York, USA

Recent epidemiological studies suggest that long-term exposure to particulate matter (PM) causes chronic effects on the cardiovascular system that result in cumulative increases cardiovascular morbidity and mortality. Since atherosclerosis is a progressive irreversible condition and an underlying cause of many cardiovascular diseases, we hypothesized that long-term exposure to PM causes adverse cardiovascular effects by exacerbating atherosclerosis. In this study, we exposed C57- and ApoE-deficient (ApoE$^{-/-}$) and ApoE, LDLr (DK)-deficient mice to concentrated ambient PM$_{2.5}$ for 6 h/day, 5 days/wk, for up to 5 mo. The overall mean exposure concentration for these groups of animals was 110 $\mu$g/m$^3$. The cross-sectional area of the aorta root of DK mice was examined morphologically using confocal microscopy for the severity of lesion, extent of cellularity, and lipid contents. Aortas from the arch to the iliac bifurcations were also sectioned longitudinally and lesion areas were stained with Sudan IV. All DK mice regardless of exposure had developed extensive lesions in the aortic sinus regions, with lesion areas that covered more than 79% of the total area. In male DK mice, the lesion areas in the aortic sinus regions appeared to be enhanced by concentrated ambient particles (CAPs), with changes approaching statistical significance ($p = .06$). In addition, plaque cellularity was increased by 28% ($p = .014$, combined), whereas there were no CAPs-associated changes in the lipid content in these mice. When examining the entire aorta opened longitudinally, both the ApoE$^{-/-}$ and DK mice had prominent areas of severe atherosclerosis covering 40% or more of the luminal surface. Visual examination of all images suggested that plaques tend to form in clusters concentrating near the aortic arch and the iliac bifurcations. Quantitative measurements showed that CAPs exposure increased the percentage of aortic intimal surface covered by grossly discernible atherosclerotic lesion by 57% in the ApoE$^{-/-}$ mice ($p = .03$). Changes produced by CAPs in male (10% increase) or female DK mice (8% decrease) were not statistically significant. In this study, we have demonstrated that subchronic exposure to CAPs in mice prone to develop atherosclerotic lesions had a significant impact on the size, severity, and composition of aortic plaque.

A very recent study on the largest cohort studied to date demonstrates that long-term exposure to fine particulate air pollution (PM$_{2.5}$) is associated with increased risk of death from cardiopulmonary diseases via mechanisms involving inflammation, accelerated atherosclerosis, and altered cardiac autonomic function (Pope et al., 2004). Other recent studies have shown that changes in blood viscosity (Peters et al., 1997), changes in heart rate (HR) (Pope et al., 1999; Gold et al., 2000), decrease in heart-rate variability (HRV) (Liao, 1999; Pope & Dockery, 1999; Gold et al., 2000), increased defibrillator discharges (Peters et al., 2000), and increased the risk of onset of myocardial infarction (MI) (Peters et al., 2001) are all associated with...
and had access to food and water ad libitum. The other mice were on a regular diet throughout, and they were switched to a normal diet 3 mo prior to the CAPs exposure. Severe skin irritation developed in some of these mice, (Adjust Calories Diet, TD88137, Harlan, Indianapolis, IN) for LDLr−/− mice lacking the low-density lipoprotein receptor (ApoE−/−−/−, obtained as breeding pairs from Jackson Laboratory, Taconic Europe, Demark) as well as double knockout (DK) mice deficient in both ApoE−/−−/− and a double knockout (DK) mice deficient in both ApoE−/−−/−−/− mice were fed a high-fat diet (Adjust Calories Diet, TD88137, Harlan, Indianapolis, IN) for 4 mo. Severe skin irritation developed in some of these mice, and they were switched to a normal diet 3 mo prior to the CAPs exposures. The other mice were on a regular diet throughout, and had access to food and water ad libitum.

**EXPERIMENTAL PLAN**

**Animals**

In this study, mice lacking apolipoprotein E (ApoE−/−−/−, Taconic Europe, Denmark) as well as double knockout (DK) mice lacking the low-density lipoprotein receptor (ApoE−/−−/−−/−, obtained as breeding pairs from Jackson Laboratory, Bar Harbor, ME) were exposed to CAPs for up to 5 mo. Animals were housed two to a cage in our ALAAC-accredited animal housing facility at Tuxedo, NY. Starting at 7 mo before the start of the CAPs exposures, ApoE−/−−/− mice were fed a high-fat diet (Adjust Calories Diet, TD88137, Harlan, Indianapolis, IN) for 4 mo. Severe skin irritation developed in some of these mice, and they were switched to a normal diet 3 mo prior to the CAPs exposures. The other mice were on a regular diet throughout, and had access to food and water ad libitum.

Fibrous plaques were examined with a computerized digital imaging system to characterize, quantitatively, size (Palinski et al., 1994) and compositional changes (Wadsworth et al., 2002) in plaques in ApoE−/−−/− mice exposed to concentrated ambient particles (CAPs). In this study, we used a computerized digital imaging system to characterize, quantitatively, size (Palinski et al., 1994) and compositional changes (Wadsworth et al., 2002) in plaques in ApoE−/−−/− mice and a double knockout (DK) mice deficient in both ApoE−/−−/−−/− mice used a computerized digital imaging system to character-

**Histopathological Evaluation of the Heart Tissues**

Hearts from air or CAPs-exposed mice were fixed with neutral buffered formalin. The atria were removed for frozen sections and the remaining ventricular tissue was embedded in paraffin. Two transverse serial sections were cut from each heart and stained with hematoxylin and eosin. The slides were coded and read blind in random order. Coronary artery profiles in the wall of the left ventricle (excluding the septum) were scored as normal or abnormal. The presence of fibrosis indicating an old myocardial infarction was also noted. All slides with one or more abnormal coronary artery profiles were reread to score the severity and extent of coronary artery lipid deposition.

**Histopathological Evaluation of the Atherosclerotic Lesions**

**Fixation, Processing, and Sectioning**

Cross-sectional atherosclerotic lesions at the aorta root regions of the heart were performed at Dr. Taatjes’s University of Vermont facilities. Mouse heart and aorta were dissected out and immersion fixed overnight in 3% parafomaldehyde/phosphate-buffered saline (PBS) at 4°C as described by Dr. Taatjes previously (Wadsworth et al., 2002). The hearts were bisected with a cut parallel to both atria infiltrated with 10% gelatin overnight at 42°C, embedded with frozen tissue embedded media (Histological stains included hematoxylin and eosin, and other stains as needed.

**Exposure to CAPs**

Animals were exposed to CAPs composed of the northeastern regional background at the New York University (NYU) A. J. Lanza Laboratory, which is located within Sterling Forest State Park in Tuxedo, NY. The CAPs were generated using a modified VACES system developed by Sioutas et al. (1999). The detailed design and performance of the entire system, as well as exposure atmosphere characterization, are described elsewhere in this special issue of Inhalation Toxicology (Maciejczyk et al., 2005). The Sterling Forest laboratory in Tuxedo, NY, is 40 miles northwest of Manhattan. Animals were exposed filtered air or CAPs at 10× of ambient concentrations for 6 h/day, 5 days/wk, for up to 5 mo. The ages and weights of these mice at the start of the exposure sequence are shown in Table 1. Exposures to CAPs were stopped on 9/5/03 and all animals were sacrificed between 9/8/03 and 9/11/03. Table 1 also summarizes mean daily parameters measured in this study.

**Table 1**

<table>
<thead>
<tr>
<th>Start date</th>
<th>Strain</th>
<th>Avg. age</th>
<th>Number of animals</th>
<th>Avg. weight (g)</th>
<th>Exposure concentration (μg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/10/03</td>
<td>C57</td>
<td>26–28 wk</td>
<td>Filtered air; 5 CAPs: 4</td>
<td>29.23</td>
<td>110 ± 79</td>
</tr>
<tr>
<td>4/10/03</td>
<td>ApoE−/−−/−</td>
<td>39–41 wk</td>
<td>Filtered air; 6 CAPs: 9</td>
<td>30.55</td>
<td>120 ± 90</td>
</tr>
<tr>
<td>5/12/03</td>
<td>DK male</td>
<td>18–20 wk</td>
<td>Filtered air; 12 CAPs: 12</td>
<td>30.29</td>
<td>131 ± 99</td>
</tr>
<tr>
<td>5/12/03</td>
<td>DK female</td>
<td>18–20 wk</td>
<td>Filtered air; 12 CAPs: 12</td>
<td>27.05</td>
<td>131 ± 99</td>
</tr>
</tbody>
</table>
Prep, Fisher Scientific), and snap frozen in 2-methylbutane cooled in liquid nitrogen (LN₂) and stored at –80°C until the time of cryostat sectioning.

Histo Prep-embedded tissue blocks were sectioned in a cryostat using disposable knives based upon the technique of Paigen and coworkers (Paigen et al., 1987). Beginning at the aortic sinus area, 10-µm sections were collected onto Fisher Superfrost Plus-coated slides as follows: Six slides each hold five step-sections collected over a well-defined 320-µm area of interest. This area was defined by three prominent valve cusps at the juncture of the aortic sinus region to the end of the valve region, when the valves disappear and the aorta becomes more rounded in appearance. The first slide held sections 1, 7, 13, 19, and 25. The second slide held sections 2, 8, 14, 20, and 26, and the third through sixth slides continue in the same fashion. Several additional single section slides were collected at the end of the 300-µm area to act as controls for subsequent staining procedures. Finally, the sections on the slides were air-dried for 30 min to ensure proper adhesion and stored in a slide box at –80°C.

Digital Image Capture and Computer-Assisted Image Processing

Consecutive sections were stained with oil red O to measure lipid content and SYTOX Green for nucleus to measure cellularity, respectively (Wadsworth et al., 2002). An Optronics MagnaFire digital camera was used to capture 1280 × 1024 pixel RGB images for SYTOX green using a fluorescent microscope. Images were cropped to isolate the artery and stitched together using PanaVue Image Assembler software. The ratio of the area stained by each stain to the total area was calculated; the number of cells stained with SYTOX/area was obtained. Quantitative analysis was performed using these values.

Evaluation of the Plaque Size of Longitudinal Sections of Aorta

Using the methods described by Palinski et al. (1994), the aorta was dissected at NYU from the aortic valve (where Dr. Taatjes took his samples) to the iliac bifurcation. The aortic tree was opened longitudinally with extremely fine microscissors (Fine Science Tools) and pinned flat on a black wax surface in a dissecting pan with 0.2-mm-diameter stainless-steel pins (Fine Science Tools). The aorta was then stained with Sudan IV (a fat-soluble dye, which stains triglycerides and protein bound lipids red) for 5 min and rinsed. Images of the aorta were captured using a Kodak Science KS290 digital photography system through a dissecting scope. Because of its length, two images were taken for each aorta and stitched together using Adobe Photoshop software. The pins and the surrounding black wax background were removed using Photoshop retouching tools for subsequent quantitative image analysis using NIH Image 1.62. The density slide tool of NIH Image was used to isolate and calculate the area stained with Sudan IV using the red channel of the acquired image. In addition to DK mice, aortas of ApoE⁻/⁻ mice were also processed and the size of the plaques was measured in the same manner.

RESULTS

During the course of the exposure, 20 deaths, all in the DK mouse, occurred in the Air and CAPs-exposed groups. As shown in Figure 1, using a log-rank test, CAPs did not affect the end of study survival in DK mouse. However, it appeared that, for the DK mice, CAPs-exposed animals died earlier than the filtered air controls. Among the 20 animals that were found dead during the course of the experiment (9 in filtered air controls, 11 in the CAPs-exposed group), 6 had good quality heart tissue sections.
by plaque in the abnormal coronary arteries were 1.0, 0.25, and 0.25, respectively. In the 7 CAPs-exposed DK mice, 3 had 0.25 of the luminal surface affected by plaque, while in the remaining 4 DK mice, the area affected by abnormal coronary arteries reached 50%. Of particular note, complex atherosclerotic lesions were seen only in mice exposed to CAPs.

In many cases only one coronary artery profile was abnormal and the other branch or branches were normal. We did not attempt to adjust the data for the number of coronary arteries seen per animal because CAD is known to be very focal.

Figures 2–4 show the lesion areas, cellularity, and lipid contents of aortas of DK mice after exposures to filtered air or CAPs for 5 mo. All animals regardless of exposure had developed extensive lesions in the aortic sinus regions, with lesion areas that covered more than 79% of the total area. These images clearly showed the formation of atherosclerotic plaques protruded into the aorta lumens. Although the lesion areas appeared to be enhanced by CAPs, with changes in male DK mice approaching statistical significance (p = .06, Figure 2), the differences in the increased lesion areas were probably functionally insignificant. In addition, plaque cellularity was increased by 28% (p = .014, combined, Figure 3), whereas there was no CAPs-associated changes in the lipid content in these mice (Figure 4).

After the aorta tree was opened longitudinally, digital images were obtained and processed to measure the size of the plaques. Figure 5 showed the original and retouched digital images, respectively, of the aorta of an ApoE−/− mouse after a 5-mo exposure to CAPs. Plaques in the hyperlipidemic mice stained red with Sudan IV were clearly visible, and were distributed throughout the aortic tree. Both the ApoE−/− (Figure 5) and DK (Figure 6) mice had prominent areas of severe atherosclerosis covering 40% or more of the luminal surface. Visual examination of all images suggested that plaques tend to form in clusters concentrating near the aortic arch (toward the left size of the image in Figure 5a) and the iliac bifurcations. The aortas of the Air and CAPs-exposed C57Bl/6 mice were devoid of vascular lesions with the exception of a few small fatty streaks (shown in Figure 7). Quantitative measurements showed that CAPs exposure increased the percentage of aortic intimal surface covered by grossly discernible atherosclerotic lesion by 57% in the ApoE−/− mice (p = .03). Changes produced by CAPs in male (10% increase) or female DK mice (8% decrease) were not statistically significant. These data were shown in Figure 8. Since the image analysis was performed on two-dimensional longitudinal images, and since plaques were clearly protruding inward, this technique would underestimate the increase in volume of the plaques in the CAPs-exposed ApoE−/− mice.

**DISCUSSION**

A major aim of this aspect of the overall study was to test the hypothesis that subchronic exposure to CAPs exacerbates the progression of atherosclerotic lesions. Our data showed that long-term inhalation exposure to CAPS-enhanced lesion cellularity and the area of aorta surface covered by atherosclerotic

---

**TABLE 2**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Air</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>DK female</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>DK male</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Number of mice with coronary artery disease

<table>
<thead>
<tr>
<th>Mice with coronary artery disease</th>
<th>Air</th>
<th>CAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>with any coronary artery disease</td>
<td>3/13 (2 F, 1 M)</td>
<td>7/10 (2 F, 5 M)</td>
</tr>
<tr>
<td>with old MI</td>
<td>2/13 (1 F, 1 M)</td>
<td>1/10 (1 M)</td>
</tr>
<tr>
<td>with complex atherosclerotic lesions in the coronary arteries</td>
<td>0/13</td>
<td>3/10 (3 M)</td>
</tr>
</tbody>
</table>
FIG. 2. Lesion areas (mean ± SE) of aorta root in DK mice exposed to CAPs. Asterisk indicates significant at $p = .06$. Bar at the bottom equals 500 µm.

FIG. 3. Cellularity (mean ± SE) of aorta root in DK mice exposed to CAPs. Asterisk indicates significant at $p = .04$. When data of male and female were combined, difference significant at $p = .01$ between air- and CAPs-exposed animals. Bar at the bottom equals 500 µm.
plagues in animals genetically prone to plaque development. Our observations were consistent with other studies showing that exposure of ApoE<sup>−/−</sup> mice to sidestream tobacco smoke (STS) exacerbated aortic atherosclerosis. Gairola and colleagues reported that exposure of ApoE<sup>−/−</sup> mice to STS for 6 h/days, 5 days/wk, for 14 wk, caused a significant increase in the percentage of aortic luminal surface covered by grossly discernible atherosclerotic lesion (from 10% in the controls to 33% in STS-exposed mice, reflecting a 2.3× increase in lesion-covered area) (Gairola et al., 2001). In this study, the area covered by the lesions increased to 66% in CAPs-exposed ApoE<sup>−/−</sup> mice versus...
42% for air-exposed ApoE−/− mice, a net increase of 57%. Since the STS exposure was performed at a much higher concentration of 25 mg/m³, our results suggested that, based on the exposure mass concentration and exposure duration, it would appear that ambient PM is considerably more potent than sidestream smoke in its ability to enhance atherosclerosis. The results of our study are also consistent with the results from another study that examined effects of CAPs on atherosclerosis in hyperlipidemic rabbits (Suwa et al., 2002). In this study, installation of 5 mg of urban PM₁₀ collected in 1993 over Ottawa, Canada, twice a week for 4 wk, produced a 70% increase in volume as well as progression toward a more advanced phenotype of atherosclerotic lesions. Although high-dose instillation studies are useful for exploratory studies, it is important for the findings to be replicated by inhalation exposure to real-world concentrations. Our study shows that long-term inhalation exposure to a real concentration of CAPs can exacerbate atherosclerosis in a widely used animal model of human cardiovascular disease.

Our study provides evidence for a biologically plausible mechanism linking excess cardiovascular mortality to chronic effects of elevated PM, namely, that PM increases the rate of atherosclerotic plaque formation. This raises a new question—What are the molecular mechanisms for the effects of inhaled PM on atherogenesis? Atherosclerosis is an inflammatory disease involving infiltration of leukocytes, accumulation of macrophages and foam cells, proliferation of smooth muscle cells, and accumulation of extracellular matrix and lipid (Libby, 2003). There is ample evidence implicating oxidative injury to the vasculature with subsequent inflammation that promotes the development and exacerbation of atherosclerosis (Uchida, 1999, 2003; Parola et al., 1999). Ambient PM contains transition metals, aldehydes, and semiquinones, which are known to undergo redox cycling and ultimately produce biologically damaging hydroxyl radicals (Dellinger et al., 2001; Ghio, 2004; Shi et al., 2003; Gonzalez-Flecha, 2004). These radicals and other pro-oxidant agents can initiate lipid peroxidation to produce reactive lipid radicals (Dellinger et al., 2001; Ghio, 2004; Shi et al., 2003; Dellinger, 2001). As discussed earlier, repeated instillation to ambient Ottawa PM₁₀ had been shown to induce a systemic inflammatory response through mediators released from the lung that stimulate the bone marrow to accelerate the release of immature polymorphonuclear leukocytes (PMN), PMN turnover in the bone marrow, and the bone-marrow pool of myeloid cells (Mukae et al., 2001). In a subsequent study, exposure to the same PM₁₀ also caused progression of atherosclerotic lesions toward a more advanced phenotype (Suwa et al., 2002) and an increase in the transit time of monocytes through the bone marrow (Goto et al., 2004). Monocytes released into the circulation can adhere to arterial endothelium and migrate into the atherosclerotic lesions, where they become lipid-laden foam cells (Libby et al., 2002; Suwa et al., 2002; Young et al., 2002; Libby, 2003). In the present study, increased cellularity was observed in DK mice aorta (Figure 3) without accompanied increases in lipid content (Figure 4) or lesion area (Figure 2). One of the possible reasons that ambient PM did not enhance lesion area of lipid content is that the advanced disease status in these mice was masking the response to PM. This is supported by the observation that a number of DK mice died in the course of this study and that severe coronary artery disease was observed in the majority of the remaining DK mice. In future studies, younger animals will be used so that it will be easier to differentiate progression of plaque between CAPs-exposed and air-sham-exposed controls with limited baseline plaque density in the aorta.

In summary, we have demonstrated that subchronic exposure to CAPs in mice prone to develop atherosclerotic lesions had a significant impact on the size, severity, and composition of aortic plaque.

REFERENCES


