

ABSTRACT

Particulate air pollution is associated with cardiovascular morbidity and mortality in epidemiological studies. Our laboratory has pioneered the development of the ambient particle concentrator as a means to carry out inhalation toxicological assessments of responses to ambient particles. Normal and compromised animal exposures to concentrated air particles (CAPs) in Boston have produced consistent and reproducible findings of biologic importance. Morphometric evidence of vasoconstriction, increases in reactive oxygen species in the heart and lungs, and increases in severity of myocardial ischemia during acute coronary artery occlusion are notable examples.

The specific objectives of this project are:

1. To differentiate the cardiovascular effects of locally emitted particles from those of transported particles using normal animals, and;
2. To determine whether spontaneously hypertensive rats, a genetically susceptible population, have enhanced vascular responses to exposures of different particle sources as compared to normal animals.

To differentiate the toxicological effects of locally emitted and transported particles on important cardiovascular outcomes, short term animal exposures to CAPs will be conducted during the time periods of 6-10am and 11am-3pm. Starting inhalation exposures at 6am before significant vertical mixing takes place will allow us to capture particles mostly from local sources. In contrast, exposures starting at 11am will be relatively more enriched in transported particles. All outcomes will be assessed in relation to those of filtered air (sham) exposures as well as those of positive controls using particles of known toxicity at both time periods to control for circadian variations. Animal exposures will be characterized using continuous measurements of particle mass, size, number, and black carbon, as well as integrated measurements of particle mass, sulfate, elements and organics.

The outcomes to be measured in the assessments include pathologic, functional, and molecular parameters of vascular response. Specific measurements will include: indicators of pulmonary and systemic inflammation, blood pressure, endothelin-1, endothelial nitric oxide synthase, atrial natriuretic peptide, *in vivo* oxidant responses in the heart and lung, and quantitative morphology of lung and cardiac vessels. These biological outcomes are critical in our efforts to investigate the mechanisms of ambient particle effects.

Statistical analyses will use multi-way ANOVA to assess differences among exposure groups and interactions of exposure and potential effect modifiers. Regression techniques will be used to examine dose-response relationships between measured biological outcomes and particle source contributions as reflected by particle composition. Multiple linear regression using tracer elements will be used to assess the independent effects of multiple pollution sources. This combination of exposure scenarios and cardiovascular outcomes will provide new data to assess the effect of specific particle sources on specific mechanistic pathways by which ambient air particles cause adverse health effects.

1. OBJECTIVES/HYPOTHESES

The goal of this project is to compare the toxic effects of locally emitted and transported particles on important cardiovascular outcomes in animals using exposures to concentrated ambient particles (CAPs). Using early morning particle exposures (mostly associated with local emissions) and mid-day particle exposures (mostly associated with transported emissions), the differences in cellular, molecular, and pathophysiological responses will be assessed as a means to define the mechanisms involved in particle-related cardiovascular responses. We postulate that associations between particle exposures and cardiovascular outcomes will differ by particle characteristics, and that local particles rich in traffic-derived carbonaceous components will have enhanced inflammatory responses and outcomes consistent with vasoconstrictive responses. We further postulate that a genetically susceptible population, spontaneously hypertensive (SH) rats, will exhibit enhanced responses compared to normal rats, especially to early morning particle exposures for all outcomes.

The specific objectives of this project are:

- To differentiate the cardiovascular effects of locally emitted ambient particles from those of transported ambient particles using normal animals, and;
- To determine whether spontaneously hypertensive rats, a genetically susceptible population, have enhanced vascular responses to exposures of different particle sources as compared to normal animals.

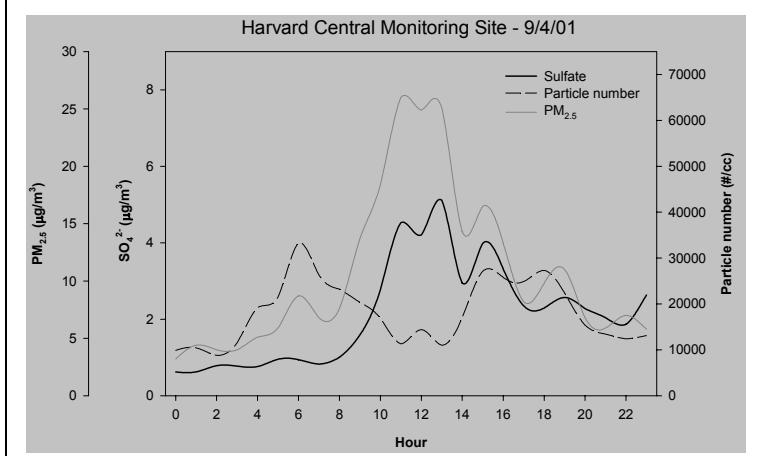
Particle exposures will be characterized using continuous measurements of particle mass, size, black carbon, and number, as well as integrated measurements of particle mass, sulfate, elements, and organics. Biological outcomes to be measured include: indicators of pulmonary and systemic inflammation, blood pressure by telemetry, endothelin-1, endothelial nitric oxide synthase, atrial natriuretic peptide (ANP), *in vivo* oxidant responses in the heart and lung by *in vivo* chemiluminescence, and quantitative morphology of lung and cardiac vessels.

2. INTRODUCTION

2.1. Novel exposure scenarios of this project: Our laboratory pioneered the development of the ambient particle concentrator as a means to carry out inhalation toxicological assessments of responses to ambient particulate matter (PM). Concentrator studies from our laboratory and others around the world have made a substantial contribution to the PM health effects field. In our laboratory using the Harvard Ambient Particle Concentrator (HAPC), we are able to increase particle concentrations by a factor of 30-40 for our studies¹⁻³.

In this project, we plan to use the typical daily concentration patterns of locally derived and transported ambient particles as a means to amplify compositional differences in CAPs exposures. Monitoring studies have shown that concentrations of specific PM_{2.5} components vary considerably over a 24-hr period⁴⁻⁶. Results from our South Boston PM characterization study showed that both particle mass and composition exhibited substantial diurnal variability. Black carbon (BC), particle number (PN) and carbon monoxide (CO) concentrations (markers of mobile source emissions⁷) were found to be higher during the morning. Evening concentration peaks of these air pollution parameters were less pronounced, which was likely due to better vertical mixing and the lower impact of diesel vehicles during the afternoon rush hour⁴. Figure 1

Figure 1: Diurnal Variation in ambient air measurements suggesting different sources of particles at specific times of day



shows the characteristic diurnal patterns for total $PM_{2.5}$, SO_4^{2-} and PN concentrations, measured at our HSPH Boston ambient monitoring site. In agreement with the results of Oh⁴, PN levels peak during the early morning and late afternoon rush hours. In contrast, SO_4^{2-} levels are highest during the mid-afternoon, reflecting the impact of regional pollution transport due to enhanced atmospheric mixing⁸.

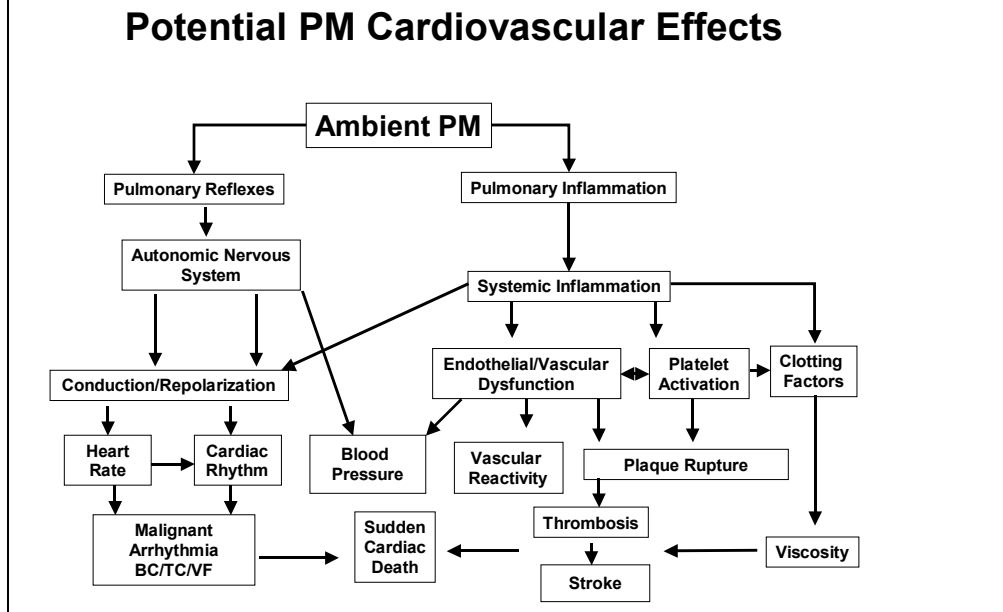
We plan to use these diurnal concentration patterns to amplify compositional differences in CAPs exposures. Starting 4-hr inhalation

exposures at 6am before significant vertical mixing takes place will allow us to capture particles mostly from local sources. These exposures will be compared to 11am-3pm CAPs exposures enriched in transported particles. All CAPs exposures will also be compared to corresponding sham (filtered air) exposures from both the 6-10am and 11am-3pm periods. To further control for circadian influences, positive control exposures of residual oil fly ash (ROFA) will be conducted at both time periods. All CAPs exposures will be characterized using a large array of measurements including continuous measurement of particle mass, PN, and BC, as well as integrated measurements of particle mass, elements, and organics as described in the Particle Technology and Monitoring Core. By comparing animal responses to these two distinct exposure scenarios on given days, we expect to differentiate toxicity of local and transported particles.

2.2. Ambient PM initiates adverse cardiovascular health effects in human and animal assessments: Evidence that ambient PM can affect cardiovascular health comes from studies that show: i) associations between daily changes in PM concentration and cardiovascular deaths and hospitalization admissions, and; ii) increased adult cardiac and pulmonary mortality associated with spatial differences in PM concentrations^{9,10} (reviewed in^{11,12}). Pathways that have been suggested as potential mechanisms to explain these associations are shown schematically in Figure 2. The first pathway involves altered cardiac autonomic function resulting from particle inhalation. Studies have observed that changes in cardiac autonomic function, as measured by heart rate variability (HRV), are predictors of cardiovascular disease and mortality¹³. Environmental epidemiological studies also report associations between the same HRV predictors and air pollution¹⁴⁻¹⁶. The second mechanistic pathway involves lung and systemic inflammation leading to vascular dysfunction. Inhaled PM may provoke a low-grade inflammatory response in the lung, release potentially harmful cytokines into the blood, induce changes in platelets and blood coagulation, increase vascular reactivity, and trigger acute cardiovascular events specifically associated with ischemic heart disease¹⁷⁻²¹.

A number of CAPs studies by our group and others have shown significant health effects supporting these pathways²²⁻²⁷. The most consistent responses to CAPs in our laboratory have been increases in the severity of myocardial ischemia during acute coronary artery occlusion^{22,28}.

Figure 2. Potential mechanisms of cardiovascular responses to ambient PM
 Modified from presentation by R. Devlin, US EPA. Society of Toxicology, 2003.



This response was found to be statistically significant in binary assessments comparing CAPs and sham exposures.

The suggested mechanism for these observations involves vasoconstriction of cardiac arteries as a result of PM exposure. Our studies in rats²⁹⁻³¹, showing changes in mediators of vasoactive balance as well as morphometric measures of vasoconstriction in the lung and heart, indicate that blood vessels are an important target of ambient PM health effects. Studies in canines^{32,33} also show morphologic changes in lung and cardiac vessels. Our Canadian collaborators have demonstrated significant findings in vascular responses in healthy adults in both brachial artery diameter and diastolic blood pressure with exposure to CAPs plus ozone³⁴⁻³⁶. Studies from our existing EPA Center show that diabetics have greater brachial artery diameter responses from increased exposure to ambient particles³⁷. Together these findings provide the direction for the proposed project, which is intended to expand our understanding of PM effects on the cardiovascular system.

2.3. Ambient particles affect molecular mechanisms of vascular responses: Increases in vasoconstrictive mediators associated with PM exposures have been shown in rats. Rats exposed to ambient particles from Ottawa, Canada, demonstrated increased plasma levels of endothelin-1 along with histological changes consistent with lung repair and remodeling^{38,39}. Studies in pigs have shown that endothelin-1 produces myocardial ischemia and ventricular arrhythmias via a potent coronary vasoconstrictive effect⁴⁰. Recent studies have shown endothelin increases in human volunteers exposed to CAPs and ozone⁴¹. Studies from our laboratory in collaboration with Dr. Renaud Vincent have shown increases in circulating endothelin in normal dogs exposed to CAPs. A sensitive rabbit model instilled with substantial doses of ambient particles showed progression of atherosclerosis¹⁸. Our micro-array studies also provide evidence of an increase in endothelin gene expression in the lung and a substantial decrease in endothelial nitric oxide

synthase (eNOS) expression with CAPs exposure, suggesting that PM may affect both sides of vasoactive balance toward vaso-constriction²⁹. However, some CAPs studies measuring blood pressure have shown little or no effect on blood pressure⁴². A possible explanation for these findings may be found in our cardiac gene expression micro-array data (presented in the preliminary studies section) in which we demonstrate a very large increase in ANP, which is a major mediator that limits blood pressure increases^{43,44}. Thus, data exist in support of several specific molecular mechanisms in relationship to vascular responses resulting from PM exposures. The proposed research will use these mechanistic outcomes to define PM effects on these mediators as they relate to specific particle components and/or sources.

2.4. Implications of these responses on systemic and cardiac vasculature: To date, findings from our animal PM exposure studies suggest heightened ischemic risk both for arrhythmias and increased infarct size during acute vessel obstruction. Human studies that demonstrate associations between increases in ambient PM and the onset of myocardial infarction⁴⁵ underscore the importance of these findings. Associations of increased ambient PM with changes in ST-segments in people with cardiovascular disease have also been reported⁴⁶. In a recent epidemiological study comparing PM-related relative risks for all cardiovascular and respiratory diseases, Pope *et al.*⁴⁷ clearly show that chronic exposures had the greatest relative risk for ischemic heart disease in all subjects. When the data were stratified by smoking status, the most dramatic increase in relative risk was found in smokers with hypertension, suggesting a synergistic response in PM-related mortality between smoking and hypertension. Other studies from our existing Center also show relationships between increases in blood pressure and increases in ambient particulate pollution^{48,49}. The proposed research will be critical in our efforts to identify the underlying molecular mechanisms of PM effects on the vasculature. Our studies will focus on examining the molecular mechanisms associated with vascular responses *in vivo*. In this project we will conduct physiological measurements similar to those measured in the human CAPs study (Project 3) and the Normative Aging Study (Project 1). In addition, we will perform important molecular measurements that cannot be done in these human studies. The interactions between investigators participating in human and animal studies in the Center will be important and will enable us to enhance our understanding of PM biological mechanisms.

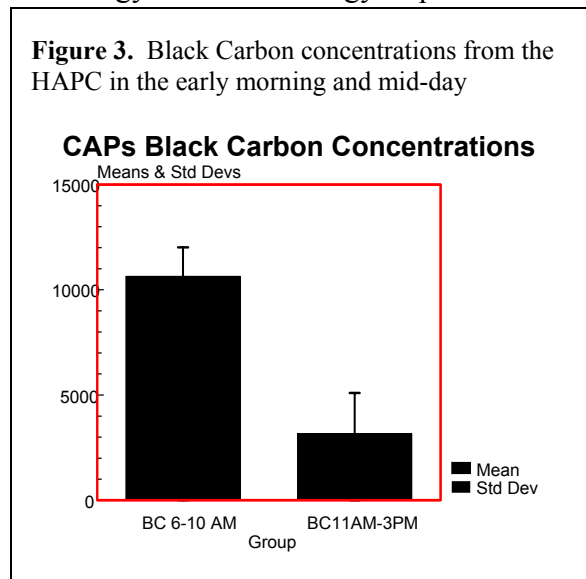
2.5. Enhanced responses in models of vascular disease: Compromised hosts are a significant susceptible population, especially individuals with chronic cardiopulmonary disease. Available models of cardiovascular disease include: a rat model of vascular injury and pulmonary hypertension induced by exposure to monocrotaline (MCT), a spontaneously hypertensive (SH) rat strain, rat models of acute myocardial infarction (MI), rabbit models of advanced atherosclerosis, and dog models of cardiac ischemia. With the rat MCT model, investigators have demonstrated ROFA-induced increased mortality, cardiac inflammation, neutrophilic pulmonary inflammation, exacerbated lung lesions, increased lung edema, alveolar thickening, and decreased phagocytosis of particles⁵⁰⁻⁵³. Others have failed to find similar inflammatory responses or changes in pulmonary function using MCT-treated rats following CAPs exposures⁵⁴. Rats pre-treated with high SO₂ exposures have been used to model chronic bronchitis and have shown interaction of CAPs with preexisting lung injury that produced changes in cellular and biochemical markers in lavage fluid and increases in tidal volume^{23,25,55,56}. In this model, significant pulmonary vascular vasoconstriction was found with CAPs exposure, but the changes were no greater in the chronic bronchitis model than those in the normal rats³⁰.

Genetic susceptibility is a research topic of great interest. Rat strains such as Sprague Dawley, Fischer-344, and Wistar demonstrate unique inflammatory and histological responses to ROFA^{57,58}. Genetically predisposed SH rats have been used to model cardiovascular disease to evaluate potential increased susceptibility to PM^{59,60}. SH rats demonstrate greater oxidative stress and cardiovascular responses than their normal counterparts in response to ROFA exposure^{60,61}. Unlike their Wistar-Kyoto strain control rats, SH rats have a compromised ability to increase bronchoalveolar lavage glutathione in response to ROFA, suggesting a potential link to increased susceptibility⁶⁰. Exposure to CAPs in a small number of SH rats resulted in increases in both blood pressure and heart rate which were statistically significant during exposures in the spring, but not significant in the summer⁶². All of these findings suggest that SH rats may be particularly useful as a model of cardiovascular susceptibility.

More complex rat models of MI have been used in our laboratory and have shown increases in arrhythmias related to ROFA and CAPs exposures^{63,64}. CAPs and carbon monoxide (35 ppm) exposures were found to have an opposite effect on arrhythmia incidence with carbon monoxide significantly decreasing ventricular premature beats⁶⁴. In contrast, preliminary studies using CAPs plus ozone (0.2 ppm) suggest synergistic effects on arrhythmia. Unfortunately, although the rat MI model^{63,64} and the canine ischemia model^{22,28} are both highly sensitive, they do not readily lend themselves to the outcomes that need to be studied or to the exposure scenario plan of this proposal. Thus, the model of susceptibility assessed in this proposal will be the SH rat. Oxidative stress will be directly measured in the heart and lungs of these animals using *in vivo* chemiluminescence as we have done previously in Sprague-Dawley rats²⁶. Most importantly, the fact that SH rats have deficiencies in responding to oxidants raises the possibility of relating the findings from this animal model to the findings of the proposed Normative Aging Study on human subjects with genetic mutations in their ability to respond to oxidants. Such interaction among projects clearly adds value to the research of this proposed Center.

3. PRELIMINARY RESULTS

3.1. Use of the HAPC and characterization of exposure: The HAPC, developed at Harvard School of Public Health^{1-3,65,66} has been a critical advance in the field of PM inhalation toxicology. This technology is proven and reliable. No surrogate material can ultimately match



ambient particles taken directly from urban air and delivered instantaneously to an experimental subject. Methods have been developed to use ambient particle monitoring techniques for characterizing concentrated ambient air samples. Subsequently, this characterization has been used to relate CAPs composition with biological response^{22,24-26,28-30}.

The utility of the HAPC for the proposed experiments is illustrated in Figure 3, which shows differences in BC concentrations at the two proposed time periods are shown. These preliminary data show the mean and standard deviation of measured BC concentrations from our fine particle concentrator. BC concentrations,

one marker of mobile source emissions, are on average much higher in the early morning as compared to mid-day. This supports our hypothesis that the impact of local emissions is likely to be greater during the early morning exposure experiments. Thus, the selected time periods for the proposed exposure experiments will be adequate to achieve the desired exposure scenarios.

3.2. Start time of experiments in our previous CAPs studies: Recently, we reviewed response data from multiple CAPs studies to explore the relationship between biological outcome and start time of exposure. Increases in severity of myocardial ischemia during acute coronary artery occlusion with CAPs exposures has been a response found consistently in binary comparisons^{22,28}. Another outcome consistently found to be significant with CAPs in binary comparisons is *in vivo* oxidant measurements by organ chemiluminescence^{26,27}. A consistently weak response was found in asthma models exposed to CAPs in our laboratory⁶⁷. On review of experiment starting times, the exposures for coronary occlusion experiments routinely began around 8am, and the oxidant experiments generally began before 9am. All exposures for the asthma studies began after 10am. Because all these experiments had distinctive start times, it is tempting to speculate that the start time resulted in specific particle component enrichments (e.g. carbonaceous versus sulfate) that influenced the results. However, because each experiment used different models with different outcomes, it is not possible to examine whether exposure characteristics or model/outcome were the determining factor for the obtained results. Furthermore, although we had one group of studies measuring the same outcomes with start times varying from 8am to noon, there were a relatively small number (6) of different experiments in this group. Although there were significant differences in composition and outcomes in relationship to the exposure start time when early and late starting experiments were compared, there were not enough data to make meaningful comparisons by regression analyses of start time, composition, and outcomes. Nevertheless, based on the review of these experiments, we formed the hypothesis that responses are related to exposure compositional differences, which are in turn related to the exposure start time. Obviously, testing this hypothesis requires experiments that measure the same biological outcomes in animals exposed during the 6-10am and 11am-3pm periods on the same days. These studies should also include detailed characterization of the exposure as well as negative and positive control animal exposures to take into account potentially important circadian variations. All of these features are incorporated in our proposed studies.

3.3. Lung inflammation and cardiac molecular responses as important outcomes to ambient PM: Our studies show that CAPs exposures produce inflammation^{24,25,27,29,55,68}. The lung responses may be augmented in the presence of underlying inflammation^{25,55,69} and the response in both normal and compromised animals is related to ambient particle composition^{24,25}. We have studied pulmonary responses to CAPs using normal animals from which we have collected total lung RNA²⁹. The RNA was pooled, labeled, and hybridized to multiple Affymetrix rat microarray chips to explore the range of responses to CAPs exposure. Using the A-chip results, data from the sham-exposed group was subtracted from the CAPs group. Since these chips typically include multiple measurements of the same gene, cluster analyses of the results as well as biologic responder cluster assessments of these micro-array studies strongly support the pro-inflammatory potential of CAPs (see Table 1). Increases in pro-inflammatory mediators such as C-C chemokines, IL-1, IL-6 and TNF are illustrated with an overall decrease in immune enhancers such as IL-2 and interferon. In addition to enhanced pro-inflammatory mediators, there is also evidence of vascular endothelial responses to inhaled CAPs. Some of these

responses are known to be concomitant with acute inflammation, but others may signal a more direct vascular response. There is also evidence in the micro-array studies for increases in reactive oxygen species activity, as well as evidence for activation of organic chemical metabolism and detoxification mechanisms.

Table 1. Micro-array studies: CAPs minus sham treated rat lung tissue RNA

Group	Genes Included	Total # of measures	Average Change
Acute Inflammatory Mediators or Receptors	IL-1, TNF, IL-6, CC and CXC chemokines IL-3, IL-18	56	↑ 75%
Immune enhancers	IL-2, IL-4, IL-7 IL-9, IL-10, IL-12, IL-13, IL-15, interferons	36	↓ 66%
Vascular activation	Endothelins, vCAM, iCAM, PAF, PECAM, p- and e-selectins	33	↑ 67%
Vascular dilatation	eNOS	4	↓ 155%
Responses to oxygen Metabolism	MnSOD, CuZnSOD, Oxygenases, Peroxidases, Cytochrome P450	78	↑ 60%
Organic chemical detoxification	Aryl hydrocarbon associated, epoxide associated enzymes	32	↑ 124%

In similarly prepared micro-array studies using RNA from the heart of the same animals described above, we found striking increases in a number of responses (See Table 2). The largest and the most interesting increase observed was in ANP. This protein functions to lower blood

Table 2. Cardiac micro-array studies: Increases measured in CAPs-treated compared to normal rat heart tissue

Group	Average
Vascular endothelial growth factor	↑ 353%
Cardiac adriamycin responsive peptide	↑ 855%
CuZn Superoxide Dismutase	↑ 1197%
Atrial Natriuretic Peptide	↑ 4020%
Cardionatin precursor	↑ 805%
Troponins	↑ 329%

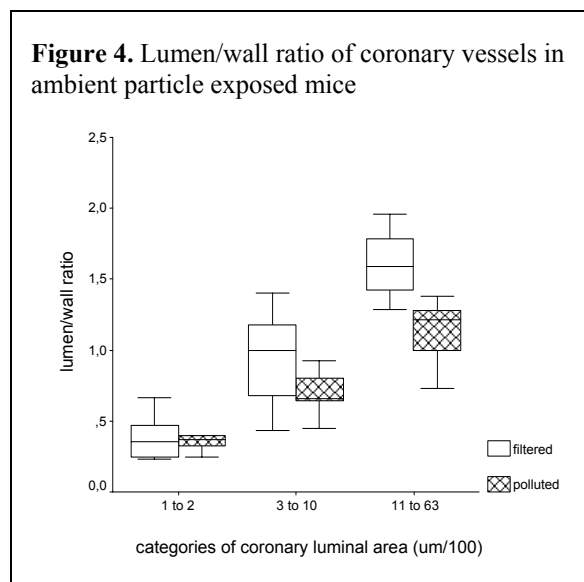
pressure, and the marked induction of this mediator may be the basis for modest changes in blood pressure in most studies. Indeed, as in the Toronto human CAPs studies, the change in blood pressure is best seen during exposure. Examinations at later times may well be influenced by increased secretion of ANP.

In this proposal, we will use quantitative real time PCR to confirm and further study the increases and decreases in gene expression found in our previous micro-array studies with CAPs. Most importantly, this quantitative molecular approach will allow us to use regression analyses to relate our gene expression findings to concentrations of locally emitted and transported particles.

3.4. *In vivo* chemiluminescence indicates oxidant responses in the lung and heart: We have shown that *in vivo* chemiluminescence is a highly sensitive measure of response to inhaled CAPs detectable in binary assessments^{26,27}. These studies correlate with the anti-oxidant responses found in the heart and lung by micro-array studies. In addition, using N-acetyl cysteine, the chemiluminescent response to CAPs can be abrogated²⁷. These studies demonstrated that the oxidant response associated with CAPs exposure plays a critical role in the initiation of the inflammatory response. Thus, it will be important to determine whether there is a difference in this lung and heart response with exposure to predominantly local versus transported particles.

3.5. Morphologic/morphometric studies of vessels in the heart and lung: In morphometric studies of the pulmonary vasculature, we found that CAPs produced a decrease in the lumen to wall ratio³⁰ that indicated vasoconstriction in the pulmonary vascular bed. In studies associating CAPs components with changes confirmed by morphometry, sulfate and silicon were found to have the strongest association. These were not the same components associated with pulmonary inflammation. In the heart, we have also observed evidence of vasoconstriction of vessels⁴³. Our micro-array studies (see Table 1 above) showed a strong increase in mediators and receptors associated with vasoconstriction and endothelial injury with an inhibition of vasodilator mediator activity. The lungs and hearts of rats exposed to CAPs or sham protocols studied by electron microscopy showed there was morphologic evidence of vascular endothelial activation throughout the lungs and heart of CAPs exposed animals⁴³. In previous studies from our laboratory, cardiac macrophages from ROFA-exposed rats contained particles that had the same elemental signature as ROFA^{2,50}. Taken together, morphologic findings provide important evidence of endothelial cell changes with exposure to ambient particles. These findings indicate that cardiovascular responses to particles include both molecular and morphological evidence of

vascular endothelial effects in both the lungs and the heart.



The same quantitative morphometric approach was used in a study of arteries categorized by size in the hearts of mice exposed to CAPs, in collaboration with Dr. Paulo Saldiva³¹. A significant decrease in the lumen/wall ratio of larger cardiac vessels in animals exposed to ambient particles was found. These results are illustrated in Figure 4. In this study, comparable assessments of another systemic vascular bed, the renal arteries, failed to show an exposure related difference in vessel sizes, suggesting that cardiac and pulmonary vessels may be specifically targeted.

4. APPROACH

4.1. Experimental Plan:

4.1.1 Objective 1: To differentiate the cardiovascular effects of locally emitted particles from those of transported particles using normal animals, and;

Hypothesis 1: Associations between particle exposures and cardiovascular outcomes will differ by particle characteristics. Local particles rich in traffic-derived carbonaceous components will have enhanced inflammatory responses and outcomes consistent with vasoconstriction.

The goal of this project is to compare the toxic effects of locally emitted and transported particles on important cardiovascular outcomes in animals using exposures to concentrated ambient particles (CAPs). Toward this end, short-term animal exposures will be conducted during the time periods of either 6-10am or 11am–3pm. Starting inhalation exposures at 6am, before

significant vertical mixing takes place, will allow us to capture particles mostly from local sources. In contrast, exposures starting at 11am will be relatively more enriched in transported particles. These CAPs exposures will be compared to concomitant filtered air sham exposures as well as exposures to CAPs from the 11am-3pm period and their corresponding sham controls. To assure that any differences observed are not due to circadian effects, positive control inhalation exposures using ROFA will also be done at both exposure periods. Furthermore, animal exposures will be characterized using continuous measurements of particle mass, size, number, and black carbon, as well as integrated measurements of particle mass and sulfate, elements, and organics. We seek to define differences in pulmonary and systemic inflammatory and vascular responses based on sources of particles in the air, and the resultant differences in composition.

We plan to compare Sprague Dawley (SD) rats exposed for 4 hrs in the early morning (starting at 6 am) with a group of similar SD rats exposed for 4 hrs starting at 11am. This strain of rat is chosen for the first objective because it has been used successfully in all our published rat studies with CAPs exposures^{25-27,29,30,43,51,55,63,64,72}. The proposed experiments for the first objective will involve CAPs and sham exposures with a total of 16 rats per group each in individual exposure chambers. The outcomes to be assessed have all been used in our laboratory group. Peripheral blood will be collected from 6 randomly selected animals from each group 24 hrs before and 24 hrs after each exposure. Complete blood count as well as plasma assessments of circulating mediators including endothelin 1, IL-6, and atrial natriuretic peptide will be performed.

We will continuously monitor breathing patterns in 6 of the animals using the Buxco system during exposure to CAPs or sham and determine the degree to which breathing parameters change during that time⁵⁵. These animals will be used in other assessments since animals in Buxco chambers are no different from animals in non-Buxco monitored exposure chambers. Bronchoalveolar lavage (BAL) will be done on 4 animals in each group 24 hrs after exposure to determine the degree of inflammation in the lung. Cell counts and differentials, as well as protein and lactic dehydrogenase (LDH) will be determined as indicators of inflammation and lung injury^{12,14,51,66}. Four animals in each group will be used for collection of RNA from the base of the heart and one lung while morphologic/morphometric assessment of lung and cardiac vessels at 24 hours after exposure^{25,30,31,43,55} will be done from the cardiac ventricles and the other lung. The lung and heart tissue collected for RNA will be used in quantitative real time PCR assessments^{50,70-73}. These assessments will include IL-1, endothelin 1, eNOS and ANP.

We will also monitor heart rate and blood pressure in another group of 6 animals with implanted DSI telemetry devices. This group of animals will not be used for other assessments. *In vivo* chemiluminescence of the lung and heart will be studied in 2 animals from each group immediately after exposure to assess acute oxidant exposure^{26,27}. There will be at least 6 different days of experiments. This will result in at least 6 morning and 6 mid-day experiments for a total number of at least 12 different experiments. We will use the outcome data in conjunction with the exposure characterization to determine the toxic potential of the CAPs on particular days and at the specified times of day. The overall design for the early morning exposure is schematically outlined in Table 3. The mid-day exposure will have the same numbers of animals and outcomes assessed at the same time points. The positive control ROFA experiments will also have the same numbers of animals per experiment and the same outcomes assessed, but these experiments will be repeated 3 times at each time period so that the numbers of animals in each outcome will be sufficient for analysis. ROFA exposure levels will be in the range of 500 $\mu\text{g}/\text{m}^3$ similar to those used in our previous ROFA inhalation experiments⁵⁰.

We will determine the extent to which BAL, breathing pattern parameters, oxidant response, and vascular injury correlate with each other and with specific exposure composition. The hypothesis to be tested in these studies is that systemic vasoconstriction develops with evidence of pro-inflammatory activity found in the lung, and this response is found with locally derived aerosol exposures rich in traffic-related composition.

Table 3. Numbers of outcomes assessed per group of rats in each 4 hr-exposure experiment

Treatment 6-10AM	Total # Exposed	Blood*	BP/HR Telemetry	Buxco**	In Vivo Oxidants	BAL	RNA/Morphol
CAPs	16	6	6	6	2	4	4
Sham	16	6	6	6	2	4	4
ROFA	16	6	6	6	2	4	4
When Assessed		24 hrs before 24 hrs after exposure	During Exposure	During Exposure	Immediately after Exposure	24 hrs after Exposure	24 hrs after Exposure

*Blood assessments will be done on the same animals before and after exposure; these animals will also be used in other assessments. **Buxco is non-invasive so animals can also be used for BAL and/or RNA/Morphology

4.1.2. Objective 2: To determine whether SH rats, a genetically susceptible population, have enhanced vascular responses to exposures of different particle sources as compared to normal animals.

Hypothesis 2: SH rats will exhibit enhanced responses especially to early morning particle exposures for all measured outcomes.

The differences in response between early morning (local particles) and to mid-day (transported particles) exposures will be assessed in SH rats as compared to their strain-related control Wistar-Kyoto (WK), and also to SD rat responses from objective 1. Characterization of exposure and outcomes for objective 2 will be the same as those described above for objective 1. Thus, for each early morning and mid-day experiment we will have 4 study groups, namely: 1) SH sham, 2) SH CAPs, 3) WK sham, and 4) WK CAPs. We anticipate that the SH rats will be more sensitive, and thus all outcomes will be enhanced as compared to their WK strain controls as well as the SD rats used in objective 1.

4.2. Experimental Details:

4.2.1. Statistical Assessment: For all outcomes, we will calculate descriptive statistics and histograms by exposure group, time of day of exposure, and rat cohort, as well as construct bivariate scatter plots of response against mass and elemental concentrations. Formal statistical analyses will first use multi-way ANOVA to assess differences among exposure groups and interactions of exposure and potential effect modifiers (type of day, rat cohort). Second, we will use linear regression to assess dose-response relationships between response and particle sources, as reflected by compositional concentrations. Univariate regressions will use either mass, particle number, or a single elemental concentration as the exposure metric. To assess the independent effects of multiple pollution sources, we will use multiple linear regression using tracer elements of previously defined pollution sources as predictors. This approach has been used in our previous studies^{22,24,26,28-30,64,67}. Sections 3.1.1 and 4.2.4 of the Biostatistics Core provide details.

4.2.1.1. Power Calculations: Power calculations for this proposed animal study use standard variance and power formulas for t-tests and linear regression. For these calculations, we have selected representative outcomes of pulmonary function, morphometric inflammation, and vascular response from our previous studies. Similar results are expected for other parameters in these categories. The primary outcomes used are Enhanced Pause (Penh), Neutrophil numeric density (Nn), and the ratio between the lumen and wall areas (L/W ratio) in pulmonary arteries. We estimated our power to detect effect sizes observed in similar rat studies conducted in our lab. Calculations were based on the proposed study design of 16 animals per exposure group (split according to outcome measured), two exposures per day, and at least five different exposure days in the study (even though more are proposed). This study design is then repeated for the SHR cohort of rats.

Using our existing data, we calculated i) the power to detect binary effects of CAPs versus filtered air; ii) the power to detect a dose-response relationship between outcome and an elemental concentration, and; iii) the minimal detectable interaction at 80% power between CAPs exposure and a potential effect modifier. This last calculation applies to detecting interactions of CAPs with time of exposure (morning versus afternoon) and CAPs with hypertension. The results shown in Table 4 indicate that we have good power to detect both binary CAPs versus sham comparisons, as well as compositional dose-response relationships, given the effect sizes observed in previous experiments. The table also indicates we will be able to detect modest effect modification by time of day or rat cohort.

Table 4. Power for proposed animal study (n=5 days, 2 exposures per day)

Outcome (Effect Size)*	Power		Minimal Detectable Interaction (Time of Day, Hypertension)
	CAPs v FA	Dose-Response	
Pulmonary: Penh (+40%,+21%)	99%	99%	85% of CAPs Effect
Systemic: L/W Ratio (-25%,-20%)	96%	99%	110% of CAPs Effect
Histology: Nn (4%, +12%)	92%	93%	140% of CAPs Effect

* Assumed effect sizes taken from our published data. The two effect sizes are the % increase due to CAPs and the % increase per SD increase in elemental concentration, respectively.

4.2.2. Ambient Particle Concentrators/Exposure Chamber System: This system is described in a number of publications^{2,3,22,25} and in the Particle Technology and Monitoring Core. We have two Harvard Ambient Particle Concentrators, two fully equipped large exposure chambers, and 48 individual rat plethysmograph exposure chambers. The exposure chambers are 4 x 4 x 8 ft stainless steel and glass units with controlled temperature and humidity. Rats are exposed in the individual units within each chamber. Filtered air control rats will be at the same temperature, pressure, and flow conditions. Filtered air for control exposure is outdoor air that is ducted through the laboratory to the chamber. Exposure will take place at the Harvard School of Public Health, located approximately a mile from downtown Boston, MA. The outdoor air enters the laboratory via a plenum at the second floor level connected to the HAPC. The inlet is located 75 meters from Huntington Avenue, a major Boston urban thoroughfare with traffic lights at each end of the block. No large industrial sources are in the vicinity. Ambient air to be used for these studies is typical of the Northeastern U.S. urban aerosol. Exposures will be conducted for 4-hr intervals in the morning and afternoon on individual days. Based on more than 5 years

experience with this system, the expected concentrations can range between 50 and 1,000 $\mu\text{g}/\text{m}^3$, but most of the exposure concentrations are expected to be in the range of 200 to 500 $\mu\text{g}/\text{m}^3$.

4.2.3. Physical and Chemical Characterization of Particles: Integrated and continuous sampling techniques will be used to assess pollutant concentrations during inhalation studies. These include mass, particle number, particle composition by X-ray fluorescence, organic and elemental carbon, organic carbon speciation by gas chromatography with mass spectroscopy, and endotoxin measurement⁷⁴⁻⁸³. These measurements and the specific methods used are described in our previous studies^{2,3,22,24-26,28-30} and in the Particle Technology and Monitoring Core.

4.2.4. ROFA Exposures: Inhalation experiments with ROFA have been described in previous studies⁵⁰. Briefly, the aerosol is generated from previously collected and milled ROFA using a Wright dust feeder system. The exposure system with individual plethysmograph chambers will be used with the same pressure and flow as in CAPs exposures.

4.2.5. Animals: SD rats will be obtained from Charles River Laboratories. They will be the same size, sex, and strain that we have used in previous CAPs studies. SH and their strain related control WK inbred male rats will also be obtained from Charles River. SH and WK will be at least 10 weeks old at purchase.

4.2.6. Pulmonary Function Testing: Breathing parameters are measured on all animals from each group as a means to assess acute pulmonary irritant responses. Previously calibrated whole body plethysmography chambers are used with an automated software system (Buxco Electronics, Sharon, CT). Up to 12 animals can be monitored simultaneously with this system. After ten minutes of animal acclimatization, respiratory parameters are measured continuously throughout exposure as described by Clarke *et al.*⁵⁵. Parameter measurements are calculated from flow changes in a pressure transducer connected to the plethysmograph. A rejection algorithm is automatically included in the breath-by-breath analysis. Baseline pulmonary function is measured 24 hours prior to CAPs- or filtered air-exposure. During exposures, breathing patterns are continuously measured and analyzed breath-by-breath to derive breathing rate, tidal volume, minute ventilation, as well as inspiration and expiration times, peak inspiratory and expiratory flows, and end inspiratory and expiratory pauses. These derived parameters are used to estimate the parameters Penh ($\text{Penh} = t_e/\text{RT} - 1$; where RT is the relaxation time derived from the expiratory cycle), Inspiratory Ventilatory Drive ($\text{IVD} = \text{TV}/t_i$), and Inspiratory Duty Cycle ($\text{IDC} = t_i/t_i+t_e$), measures indicative of bronchoconstriction and respiratory effort.

4.2.7. Blood Pressure and Pulse Rate: These parameters will be monitored with our DSI telemetry system using Physiotel PA-C40 transmitters and data will be acquired with Dataquest A.R.T. version 3.0. Transmitters will be surgically implanted in the abdomen of the rat at least 2 weeks before exposure. The pressure transmission catheter will be secured in the iliac/femoral artery. Systolic, diastolic, mean blood pressure and heart rate will be obtained from the continuous pressure signal from the animal. We can monitor 12 animals continuously throughout exposures. Animals with these implanted devices will be used in multiple exposure experiments in a CAPs-Sham crossover design with at least two weeks between exposures for any animal.

4.2.8. In Vivo Chemiluminescence: Spontaneous chemiluminescence of the surface of the lung and heart is a sensitive measure of reactive oxygen species in tissues. It will be monitored using a photon counter with a photomultiplier responsive in the range 300-900 nm²⁶. Anesthetized animals (Nembutal 50 mg/kg body weight) will be connected to an animal ventilator, and the

chest will be open to expose the lungs and heart. Measurements will be performed within 10-20 min in a light-tight chamber kept at 32-36 °C. Shielding of the heart or lung as the other organ is measured by aluminum foil is typically done to make both measurements in the same animal. The emission will be expressed as counts per second per unit of lung surface (cps/cm²)^{26, 84-86}.

4.2.9 Blood Collection: One day before exposure, 0.2-0.5 ml of blood will be drawn under sterile conditions from the tail vein of each animal. This blood will be stored as frozen serum and plasma samples and used for post-exposure comparisons of circulating mediators such as endothelin. Twenty-four hours after exposure, the animals are lightly anesthetized with sodium pentobarbital (5-10 mg/kg). At this time, 5 ml of blood will be collected by cardiac puncture. A portion of the blood will be used for complete blood count and peripheral blood cell differential. Of the remaining sample, half will be saved as plasma and half as serum. After blood collection, the animals are given an overdose of pentobarbital to carry out BAL or other tissue harvest. Blood will be used in analyses of circulating mediators.

4.2.10. Mediator Analyses in Blood: Endothelin-1, ANP, and IL-6 will all be analyzed in blood. All standard peptides, antibodies, and assay reagents will be obtained from a commercial radioimmunoassay kit for rat ANP from Peninsula Laboratories, Belmont, CA. Endothelin-1 assay will use the commercially available Rat Endothelin-1 enzyme-linked immunosorbent assay (ELISA) kit from Assay Designs, Ann Arbor, MI. Plasma IL-6 will be assayed using a commercially available Rat IL-6 Quantikine ELISA kit from R&D Systems (Minneapolis, MN).

4.2.11. Bronchoalveolar Lavage Studies: Pulmonary inflammatory parameters will be assessed using methods we have reported in numerous studies^{25,29,50,55,68,71-73}. Following blood collection (4.2.9) 24 hrs after exposure, animals will be euthanized and cells collected by BAL (12 lavages x 6 ml) with sterile PBS/0.6 mM Na₂ ethylene diamine tetra-acetic acid (EDTA). Fluid from the first lavage will be centrifuged briefly; the supernatant and cells will be stored separately at -4°C. Yields and viability will be determined by hemocytometer counts of aliquots diluted in trypan blue solution. Cell type will be determined from modified Wright-Giemsa-stained cytocentrifuge preparations. Remaining cells recovered by BAL will be lysed in guanidine thiocyanate and stored at -70°C for RNA isolation. **Total Protein in Lavage:** Protein concentration will be determined by the Bradford procedure using bovine serum albumen as the standard (Pierce, Rockford, IL). **Biochemical measurement of lysosomal enzymes:** Assessment of lysosomal enzymes will be carried out as we have described in previous studies^{25,29,50,55,71-73}.

4.2.12. Histopathologic, Ultrastructural and Morphometric Assessments: Morphometric assessments will quantify vasoconstriction of vessels in heart and lung tissues. The right lung will be assessed in these studies, and the left lung will be used for RNA sample analysis as described in the next section. The left bronchus will be tied off and the lung removed. The right lung will be fixed by intratracheal fixation *in situ* at a constant pressure of 20 cm H₂O with 2% glutaraldehyde in 0.085 M cacodylate buffer, 0.05% CaCl₂, pH 7.4. The lung will be removed and stored in fixative at 4°C. The heart will be dissected out and the atria and upper portion of the ventricles taken for RNA. The remainder of the ventricles will then be immersed in fixative. Morphometry will be carried out as described in our previous studies^{25,30}.

4.2.13. Quantitation of Gene Expression in Tissue Samples: Quantitative real-time RT-PCR is needed to quantify the responses obtained in micro-array studies of heart and lung tissues with CAPs exposures. Tissue from 4 rats will be obtained 24 hrs after each exposure. The left lung,

atria, and upper portion of the heart ventricles will be collected for RNA analysis. Quantitative real-time RT-PCR will be performed in our laboratory using an iCycler iQ Real Time Detection System and iQ SYBR Green Supermix in accordance with manufacturer instructions (BioRad, Hercules, CA). Data will be normalized to GAPDH mRNA. Fresh peripheral lung and cardiac tissue specimens will be frozen in a dry ice/methanol bath and stored at -80°C. Total RNA is extracted with Trizol reagent (Life Technologies) in accordance with manufacturer instructions. Samples will be aliquoted and stored at -80°C. Reverse transcription reactions will be performed after DNase treatment with 1 µg total RNA, random hexamers (Life Technologies), rRNasin ribonuclease inhibitor (Promega, Madison, WI), and MLV RT (Promega) as has been described in detail in the literature⁷⁰. RT reactions are diluted in water, aliquoted, and stored at -20°C. Each 1 µg of RNA generates enough cDNA for ~30 PCR reactions, so the amount of RNA should not impose any limits on testing. Quantitative real-time RT-PCR is performed on 4 µl cDNA using an iCycler iQ Real Time Detection System and iQ SYBR Green Supermix in a final volume of 50 µl, in accordance with the manufacture's instructions (BioRad). Primers for the rat genes of interest, namely IL-6, endothelin-1, eNOS, and ANP are readily available. The primers chosen are designed to amplify genomic regions that cross introns to limit the effects of DNA contamination. For each experiment, the threshold cycle is converted into copy number by a standard curve. Melting curve analysis follows all PCR reactions to ensure one peak on a plot of -df/dt vs. temperature. Copy number will be normalized to GAPDH copy number for each reaction. GAPDH is a suitable control, as copy number does not change significantly with or without inflammation. Dr. Eric Silverman is the director of the molecular core facility at HSPH, within Dr. Godleski's laboratory, and is responsible for administering the BioRad iCycler iQ real-time resources. Access to this equipment for the tasks of this proposal is assured.

5. EXPECTED RESULTS

These studies will delineate differences in local particles (predominantly traffic) from transported particles with respect to vascular outcomes. The amplification of particle exposures associated with local and distant sources will allow for comparisons of outcomes important in the hypothetical mechanistic pathways of ambient PM cardiovascular effects. The outcomes focus on responses important in oxidant initiation of pulmonary inflammation, mediators of vasoactive balance, and functional measures of vascular health. Thus, this project will complement the other Center projects and especially the controlled human CAPs exposures and the Normative Aging studies. These human studies are focused upon the same outcomes, but cannot directly assess the morphologic and molecular outcomes that are crucial to our understanding of particle mechanisms of cardiovascular responses. In addition, the outcomes studied in this project complement the experiments proposed in Project 5, which compare the effects of primary and aged traffic-generated aerosols from a Boston tunnel.

The detailed analyses of CAPs exposures will make it possible to characterize compositional differences between the early morning and mid-day exposures and relate them to biological response outcomes important in the pathophysiologic mechanisms of response.

A particularly intriguing observation has been made in studies of human myocardial infarction. There is a well-documented peak in myocardial infarction incidence in the early morning hours about 6am⁸⁷⁻⁹⁰. Although many hypotheses have been offered to explain this observation⁹⁰⁻⁹⁷,

most are related to the same factors implicated in mechanisms of cardiovascular ambient particle toxicity. This peak in myocardial infarction incidence corresponds very closely to the early morning peak in ambient PM. Thus, our proposed study, assessing cardiovascular outcomes in relationship to the peak in early morning particles from local sources, also has the potential to shed light on mechanisms related to this fundamental problem in cardiology.

6. GENERAL PROJECT INFORMATION

6.1. Investigators: Dr. John Godleski directs this project. He is an experimental pathologist with extensive experience in inhalation toxicology using CAPs exposures with animals. He will be responsible for all aspects of the study especially those related to cell and molecular biologic outcomes. Dr. Beatriz Gonzales-Flecha is a co-investigator who has pioneered the use of *in vivo* chemiluminescence in ambient particle research, and will have responsibility for these aspects of the study and analyses. Dr. Joy Lawrence is a co-investigator with extensive experience in operation of the HAPC and in carrying out the monitoring measurements to be done in this project. Dr. Edgar Diaz is a research associate who will be responsible for the animal exposures, pulmonary function studies, blood studies, and bronchoalveolar lavage. Dr. Gregory Wellenius is a post-doctoral fellow who will be responsible for the telemetry studies.

6.2. Animals: Rats strains selected for this project include SD(used successfully in many studies with CAPs), SH rats, and their strain-related controls, WK rats. The SH model of susceptibility is chosen in keeping with the theme of vascular assessments in the human exposure and normative aging studies. These SH rats also have a known dysfunction in handling oxidant responses so it is particularly important as a corollary to both our *in vivo* oxidant measurements and the genetic studies to assess oxidant response mutations. Harvard School of Public Health is accredited by the American Association for the Accreditation of Laboratory Animal Care and meets National Institutes of Health standards as set forth in the "Guide for the Care and Use of Laboratory Animals" DHEW Publication No. [NIH] 78-23 Revised.

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