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INHALATION TOXICOLOGY METHODS: The Generation and Characterization of Exposure Atmospheres and Inhalational Exposures

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Abstract

In this review, we outline the need for laboratory-based inhalation toxicology studies, the historical background on adverse health effects of airborne toxicants, and the benefits of advance planning for the building of analytic options into the study design to maximize the scientific gains to be derived from the investments in the study. We then discuss methods for: 1) the generation and characterization of exposure atmospheres for inhalation exposures in humans and laboratory animals; 2) their delivery and distribution into and within whole-body exposure chambers, head-only exposure chambers, face-masks, and mouthpieces or nasal catheters; 3) options for on-line functional assays during and between exposures; and 4) options for serial non-invasive assays of response. In doing so, we go beyond exposures to single agents and simple mixtures, and include methods for evaluating biological responses to complex environmental mixtures. We also emphasize that great care should be taken in the design and execution of such studies so that the scientific returns can be maximized both initially, and in follow-up utilization of archived samples of the exposure atmospheres, excreta, and tissues collected for histology.

Keywords

Aerosols; Airborne toxicants; Animal exposures; Atmosphere generation; Complex mixtures; Exposure chambers; Exposure-response; Inhalation exposures; Human exposures; Particle size; Extrapulmonary; Telemetry; Non-invasive

Introduction – The Need for Laboratory-Based Inhalation Toxicology Studies

The health-related effects of airborne toxicants can best be studied in laboratory settings where the investigators can control: 1) the nature and temporal pattern of the exposure; 2) the selection of the test subjects in terms of species, ages, gender, and known or suspected susceptibilities; 3) the nature and timing of the appropriate response assays before, during, and after the exposures. There are, of course, constraints on the freedom of the investigators to control these major categories of the determinants of the exposure-response relationships. First, there are ethical constraints imposed by Institutional Review Boards (IRBs) for human subjects, and Institutional Animal Care and Use Committees (IACUCs) for laboratory animals. Second, there are technical constraints imposed by the technological capacity of the

available hardware and methodology for the generation and monitoring of the exposure atmospheres, especially for particulate matter (PM) exposures with different particle size distributions, and/or complex environmental mixtures. Third, there are cost constraints imposed by limited funding in recent years for toxicity testing to meet regulatory requirements, and especially for research studies. These can be manifest in terms of the number of subjects, the number of time-points for acute effects assays, the duration of the exposure sequence and/or follow-up times, especially for chronic effects assays. Costs can rapidly escalate when the study calls for the use of new knockout strains of mice, multiple strains, larger animal species, and most of all, when the subjects are human volunteers. Despite these constraints, such studies are needed as a basis for gaining regulatory approvals and for establishing air quality standards, guidelines, and source control strategies that both protect the public health and minimize societal costs.

Historical Background on Adverse Health Effects of Inhalation Exposures of Airborne Toxicants

Our earliest evidence that inhalation exposures caused adverse health effects came from observational studies of the effects caused by occupational exposures to specific chemicals or mixtures in mines and ore processing mills such as smelters, metals fabrication, and chemicals and petrochemicals manufacturing and utilization. Causal inferences could be established in the absence of mechanistic understanding by consistent differences in response according to intensity of exposure to a specific agent, to a mixture, or to a marker of the mixture. Specific toxicants were associated with specific adverse effects, such as in occupational and environmental exposures to lead (Pb) and neuropathy (Grant 2009, Fischbein and Hu 2007), mercury (Hg) and neurotoxicity (Grandjean and Nielsen 2009, Goldman 2007), carbon monoxide (CO) as a cause of hypoxia and ischemic heart disease (Kleinman 2009), benzene (C₆H₆) as a cause of hepatic and bone marrow defects and of leukemia (Goldstein and Witz 2009, Landrigan 2007), silica (Si) with silicosis (Jalloul and Banks 2007), and asbestos fibers with asbestosis, lung cancer, and mesothelioma (Rom 2007, Lippmann 2014). Similarly, for some general population exposures to complex mixtures, it was shown that there were exposure-intensity related excesses of lung cancer and cardiac mortality in humans for passive cigarette smoke exposures (Samet et al. 2009) and for coal smoke related community air pollution (Thurston et al. 2013), although, in these cases, the specific constituents within the mixtures that caused the effects are not yet adequately known.

In order to go beyond these first stage investigations of causality, we need controlled inhalation exposure studies in order to determine the contributions of components of a complex mixture to the effects, and to characterize the biological mechanisms and temporal dynamics that determine and control the progression of the lesions that lead to disease expression at realistic levels of occupational and/or environmental exposures.

Planning an Experimental Inhalation Study and Building Analytic Options into the Study Design

In consideration of the limited opportunities for conducting inhalation exposure studies, and especially for chronic exposure studies, it is important that careful consideration be given to both the specific purpose(s) and objective(s) of the study sponsor, and of likely and/or hypothesized supplemental usage of the data that will be generated and the samples collected from the exposure atmospheres and the excreta and tissues of the exposed subjects. Preservation of archived data and samples can be used to address hypotheses generated by the findings of the original study and to harness new technologies that are capable of more sensitive measurements, additional analytes, and the detection of new biomarkers of biological responses. They may also be a great resource, to the original team of investigators, as well as to collaborating scientists from complementary disciplines with interests in organ systems other than those of the original investigators. The utility of such collaborations was demonstrated to us during our Health Effects Institute (HEI) supported National Particle Component Toxicity (NPACT) study, which was focused primarily on the cardiovascular effects of inhaled ambient air fine particulate matter (PM_{2.5}). We had requests for the tissues of chronically exposed mice, and the analyses of others demonstrated that there were significant responses in the brain (Veronesi et al. 2005, Gillespie et al. 2011), and liver (Tan et al. 2009), as well as those that we found in the cardiovascular system. Similar productive collaborative experiences were reported in the inhalation exposure studies on complex mixtures at the Lovelace Respiratory Research Institute (LRRI) (Seagrave et al. 2008, Mauderly 2014).

Planning for any inhalation study also needs to consider:

1. The selection of the most realistic exposure atmospheres and concentrations for meeting the study objectives.
2. The need to have extensive measurements of the exposure atmospheres to serve the needs of the original study objectives, and anticipated follow-up studies.
3. The need to collect and archive translational biological fluids (serum, lavage, urine, etc) and tissues for subsequent “omics” studies (genomics, proteomics, metabolomics, lipidomics, etc).
4. The need to set up and maintain user-friendly data files for quality control and appropriate statistical analyses.

Selection and Generation of Controlled Exposure Atmospheres for Quantitative Studies of Exposure-Response Relationships

There are a limited number of broad categories of exposure atmospheres that will be described and outlined in this chapter, i.e.,

- single component chemicals in the gas phase;
- single chemicals in particulate form in a single particle size or limited particle size range;

- simple mixtures, typically one type of particle and a single gas phase chemical; and
- source-related complex mixtures, such as engine or power plant effluents that have undergone realistic dilutions and aging times.

For each category above, a determination of an exposure-response relationship will usually require the delivery of at least three graded concentrations and a clean-air control stream. The sham exposures of a matched group of test subjects to toxicant-free air provides a control for the possible effects of undergoing the test protocols other than those caused by the deposition and retention of the inhaled toxicant. In some cases, exposures to an agent with similar physical/chemical characteristics, of the agent in question, that could elicit positive response may be warranted. The effects can then be expressed in terms of the differences in the responses to a sham exposure and those of the test agent exposures. The range of exposures, in terms of the combination of concentration and duration, are generally selected to yield one or more clear biological responses at the upper bound exposure, and few or no exposure-related responses at the lower bound exposure. The choices for the exposures for single component chemicals are simpler than those for simple mixtures, where there may be chemical reactions between the chemicals before they are inhaled, and/or potentiation of the responses due to the separate or interactive effects of the components of the mixture. For exposures to complex ambient air mixtures, where the composition of the mixture, and the concentrations of its constituents change from hour-to-hour and day-to-day, the selection of the exposure variables likely to produce responses of interest becomes even more challenging.

For exposures to mixtures at multiple concentration levels, the simplest and most convenient method for assuring constant ratios of the constituent concentrations is to produce the lower concentrations by serial dilution of the mixture at the highest concentration with a stream of clean air.

The selection of an upper bound concentration is also dependent on the physical and chemical characteristics of the test agent, and how these factors affect the dose delivery within the respiratory tract. For gases and vapors, aqueous solubility is a critical determinant. For a highly soluble vapor, such as sulfur dioxide (SO₂), there is little penetration beyond the larger airways, while for a less soluble gas such as ozone (O₃), there is much greater penetration into the smaller conductive airways and gas exchange region of the lungs and easier access to the epithelial cells.

For PM exposures, the critical variables are particle size and hygroscopicity. The penetration of inhaled particles to deposition sites in the airways is dependent on their aerodynamic diameter for particles larger than about 1 µm, which deposit by impaction and/or sedimentation, and on physical diameter for smaller particles, which deposit by diffusion. For hygroscopic particles passing through the warm and very humid respiratory airways, there is water vapor absorption and growth into solution droplets, which can greatly increase deposition by impaction and sedimentation in the larger airways, and lessen deposition by diffusion in the smaller airways. Aqueous solubility also can greatly influence dose delivery of dissolved gases and ions to sites beyond the respiratory tract through the more rapid and greater access to the bloodstream.

The selection of test agents for controlled inhalation studies needs to consider the types and conditions of the exposures and the characteristics of the test subjects, especially for clinical studies involving human subjects. Some of the important differences between humans and animals undergoing experimental exposures are:

- Humans can be exposed by either nasal or oral breathing, while most animals are obligatory nasal breathers. Oral breathing bypasses the upper respiratory tract, resulting in greater test agent penetration into the lower respiratory tract. Since the nose of rodent is an efficient particle filter, the particle size range that can be delivered to the lungs is more restricted for rodents than for humans.
- Humans have dichotomous (nearly symmetric) branching of the conductive airways in the lungs, while animals have highly asymmetric branching airways in their lungs. Thus, the bifurcations of human airways are favored deposition sites for particle impaction, and overall conductive airway deposition is greater in humans than in experimental animals (Lippmann and Schlesinger 1984).
- Humans can be cooperative subjects, and participate in protocols involving variable breathing rates and tidal volumes, as well as both nasal and oral breathing. Human subjects can also perform tests of physiological response that are less feasible for test animals. By contrast, most animal inhalation studies have been restricted to quiescent nasal breathing.
- Human studies are limited to those focused on short-term exposures and transient physiological responses. By contrast, animal studies can include long-term exposures, serial sacrifice, and pathological assays.
- The differences in: deposition efficiencies and sites within the airways; in metabolic pathways and rates; and in lifespan between the species, makes it difficult to extrapolate the effects observed in one species to those that may occur in another, especially for effects that may occur in organs beyond the lungs and late in the lifespan.

Background on the Generation and On-Line Monitoring of Controlled Gas and Vapor Exposure Atmospheres

Inhalation exposures take place at normal ambient temperatures, and gases, by definition, do not condense at ambient temperature. Thus, the simplest way to generate a low concentration of a chemically stable toxicant gas is to blend a small stream of the toxicant from a compressed gas cylinder into a much larger stream of clean air. This can be room- or outdoor-air that has passed through a high efficiency filter to remove background PM, and through beds of adsorbent granules to remove background gases and vapors that may affect the stream going into the exposure apparatus. For a highly reactive gas, such as O₃, it may be necessary to generate it on-site.

For liquids with an appreciable vapor pressure at ambient temperatures, the simplest approach is to draw off the vapor above a liquid reservoir and blend it into the airstream going to the exposure apparatus. In this case, it is important to keep the liquid reservoir at a

constant temperature in order to generate a constant concentration of the vapor. For more guidance on specific technical means of controlled atmosphere generation of gases and vapors, see Wong (1995) and/or (Moss 2001).

Continuous on-line monitoring of the concentration of most gases and/or vapors within an exposure chamber is feasible by drawing a small volume stream into the inlet of an externally located direct-reading instrument. However, for reactive chemicals, it is important to locate the sampling line inlet at a representative location within the chamber. These data can then be used as feedbacks to control the concentration with appropriate hardware and software.

Background on the Generation of Controlled Single Aerosol Exposure Atmospheres

Aerosols, by definition, are suspensions of PM in air. The complexity of the challenge in generating a test atmosphere for an inhalation exposure study arises from the extreme diversity of the physical and chemical characteristics of aerosols in occupational and environmental settings that one may want to study. Among the variety of test aerosols that can be studied, we have:

- Monodisperse aerosols, especially useful for studies of the effects of particle size on deposition efficiencies and patterns within the airways. They can be produced with a vibrating orifice or spinning disc.
- Condensation aerosols of pure materials, which are generated by vaporization of a pure material, followed by controlled condensation. Such aerosols can have a relatively narrow range of particle sizes and be spherical or crystalline in shape.
- Dry dispersion aerosols. Such aerosols are dispersions of pure materials that have been comminuted to produce dry powders that can be mechanically dispersed into a clean airstream as a dust with a well-characterized particle size distribution.
- Wet dispersion aerosols. Such aerosols are dispersions of liquid droplets that are sheared from a liquid reservoir by a spray nozzle, or a filament breakup from a vibrating orifice, rotating disc, or electrospray. The aqueous droplets may contain suspended particles e.g., monodisperse polystyrene or colloidal particles) and/or dissolved solids (e.g., salt, emulsions, etc.) and they shrink as the water evaporates. The sizes of the residual particles depend on the concentrations of the suspended particles and dissolved solids in the original droplets.

Continuous on-line monitoring of the concentration of most chemicals in particulate forms within an exposure chamber is seldom feasible because of the paucity of continuous monitors for specific particles. However, by using non-specific monitors for PM mass concentration and/or particle number concentration as a surrogate index of the concentration of the exposure agent, a chamber operator can adjust the contaminant feed rate to maintain a reasonably constant concentration within the exposure chamber.

For more on specific technical means of controlled atmosphere generation of aerosols, see Moss and Cheng (1995), Hollander (1988), and Cheng and Chen (2001).

Background on the Generation of Controlled and Reproducible Mixture Exposure Atmospheres that are Temporally Stable

Steady-state processes can generate complex mixtures that are reproducible for repetitive daily exposures that lead to transient and chronic effects in the respiratory tract and in other organ systems resulting from the translocation of soluble gases, vapors, and particles and/or the dissolution of components of the particles, and their distribution via the bloodstream. Ambient air mixtures that have been associated with adverse health effects include combustion effluents such as sidestream cigarette smoke (Chen et al. 2010), motor vehicle exhaust (Campen et al. 2013), and wood smoke (Tesfaigzi et al. 2002). These combustion effluent mixtures all contain both PM and gaseous toxicants such as NO, NO₂, SO₂, and organic vapors. Studies have also investigated the effects of other ambient air mixtures that lacked toxicant vapors, e.g. resuspended road dust (Seagrave et al. 2008), and various kinds of nano-particles (Cuevas et al. 2010, Gillespie et al. 2010, Liberda et al. 2010).

Generation of Complex Exposure Atmospheres

Coal, Residual Oil, and Wood Smoke Emission Particles

A laboratory-scale laminar-flow drop-tube furnace that we previously used for animal exposures (Chen et al. 1990) was used to produce the 3 types of combustion-emission particles for introduction into an exposure chamber. The system consists of a feeder, a laboratory scale laminar-flow drop-tube furnace, and a collection probe. Pulverized coal, sieved to 50–60 µm; #6 oil droplets, 20 µm produced by nebulization; and wood chips, sieved to 50–60 µm, have been carried in a nitrogen gas stream and injected axially downward into the furnace where the particles are ignited and burned in a narrow zone along the furnace axis. Bulk gas temperature in the isothermal combustion zone has been maintained at 1250°K. Details of the design and operation of this system were described previously (Amdur et al. 1986; Neville et al. 1983). A schematic diagram of the furnace system is shown in Figure 1.

Upon completion of combustion, all gaseous and solid products are passed through an Argon-quenched water-cooled probe, inserted axially through the bottom of the furnace. Argon gas is transpired through a porous stainless steel liner in the probe to prevent the deposition of ash particles on the furnace walls. At the exit of the collection probe, a 3-stage virtual impactor (Intox, Albuquerque, NM) removes particles with aerodynamic diameters larger than 2.5 µm and the emission particles and the carrier gas mixture are then introduced into the exposure chamber. The oxygen (O₂) content in the exposure chamber is maintained at 20% by metering 100% O₂ into the aerosol-gas mixture, and the entire effluent is fed into the exposure chamber. Carbon monoxide (CO) and SO₂ are removed using denuders prior to animal exposure.

Diesel Exhaust

We have produced whole diesel engine exhaust using a 5500-watt single-cylinder diesel-engine generator (Yanmar YDG 5500EE-6EI, Osaka, Japan) (Quan 2010). It contains a 418-cc displacement air-cooled engine, and is operated at a maximum engine load condition using #2 on-road ultra-low-sulfur diesel fuel delivered from a local gas station. Electrical current is drawn from the engine to provide a constant load (~100%) with a 5000-watt electronic heater. Engine oil (SAE, 15W/40, Delo400, Chevron) is changed every 50 hours of engine operation. The diesel-engine exhaust is diluted to a desirable level through a serial dilution system with ambient air filtered through a high efficiency filter before introduction into the exposure chamber. CO and nitrogen oxides (NO_x) are removed using denuders.

Soil-like Particles

We have produced aerosols containing soil-like particles by resuspending Arizona road dust using a fishing line system developed at EPA (Wichers et al. 2006), and have used this system to resuspend particles generated by the collapse of the World Trade Center (WTC) towers into animal inhalation exposure chambers (Vaughan et al. 2014). The general principle of this system has a cotton string pulled through a particle-filled reservoir to allow particles in a dust reservoir to loosely adhere to it. The string then passed into a 'discharge head' where filtered air blows the particles off and into a mixing chamber that served as the exposure interface. Soil particles larger than PM_{2.5} are removed by an in-line cyclone before being introduced into the exposure chamber. Alternatively, animals can be exposed to the whole output of this generator, including large supercoarse particles (>20µm) without the cyclone through intra-tracheal inhalation as described in Vaughan et al (2014). The schematic diagram of this generator is shown in Figure 2.

Aging Chamber

Emission source particles can be captured and delivered into aging ducts and/or chambers, where they can be monitored for various gas-phase and particle-phase characteristics. The aerosol stream can be diluted, mixed with a carrier gas, or filtered, depending on the desired exposure approach. Following the design of Weitkamp et al. (2007), an aging chamber can be linked with exposure chambers as been done in the TERESA studies (Godleski et al. 2011).

Concentrated Ambient Particulates (CAPs)

We have used a modified Versatile Ambient Particle Concentrator Exposure System (VACES) to produce PM_{2.5} concentrated air particles (CAPs) at multiple sites for mouse inhalation exposures (Chen et al. 2010a; Lippmann et al. 2006; Lippmann et al. 2013; Maciejczyk et al. 2005a, b; Quan et al. 2010a; Sun et al. 2005). This system allows for simultaneous exposure of up to 64 mice to CAPs, with an equal number of sham-exposed mice as controls. ECG patterns of sixteen animals per group can be monitored during the exposure period. A schematic diagram of the inhalation exposure system is shown in Figure 3.

Nano-Particles

Nanoparticles of various compositions are generated with an arc furnace using a system that was developed by Oberdorster et al. (2000), and modified for our studies (Cuevas et al. 2010; Gillespie et al. 2010; Kang et al. 2011a; Kang et al. 2011b; Liberda et al. 2010). The nanoparticles are produced by a spark discharge between opposing high purity metallic electrodes (Ni, Ag, and Ce with 99.9 to 99.995% purity, ESPI, Ashland, OR) using a Palas® GmbH arc furnace (Model GFG-1000, Karlsruhe, Germany) and transported from the furnace with an ultrapure Argon carrier gas before mixing with air prior to delivery to an inhalation exposure chamber. Our laboratory has also used a similar technique for transporting nanoparticle-size nuclei from a furnace to a dilution system and an animal exposure chamber. The particle stream of nanoparticles passes through non-conductive and charge-neutralized tubing (to reduce wall losses) and is diluted with filtered air before entering the exposure chamber (Amdur and Chen 1989; Chen et al. 1992; Gordon et al. 1992). Oxygen is added to maintain the exposure atmosphere at 21% O₂.

Mainstream and Environmental Tobacco Smoke

Environmental tobacco smoke (ETS) is composed of 85–90% sidestream smoke (SS), with the remainder being exhaled mainstream smoke (MS). In our studies, we have used SS as a surrogate for ETS. Animals were exposed to 120 µg/m³ ETS for 6 hr/d, 5 days/wk, for up to 6 months (Chen et al. 2010c).

Tobacco and Health Research Institute's 3R4F cigarettes (or the most recent replacement with similar tar and nicotine content) have been used to generate the exposure atmosphere. The reference cigarettes are stored, as suggested by the Institute, at 24°C and 60% RH. The cigarette smoke is generated with an automated cigarette-smoking machine (CH Technologies, New Jersey).

One cigarette is lighted at a time to produce MS with an automatically regulated piston pump using a two-second puff of 35 ml volume in a bell shaped profile, as specified in ISO 3308, once per minute. When the appropriate butt length is reached, the spent cigarette is ejected and a new one loaded and ignited for continuous operation. The MS and SS are diluted with filtered air and introduced into a 1.3m³ stainless steel chamber for animal exposure.

Inhalation exposure studies have also utilized laboratory-generated mixtures to represent the ambient air mixtures. The Lovelace Respiratory Research Center (LRRI), in their part of the Health Effects Institute (HEI) National Particle Component Toxicity (NPACT) Study, utilized this approach to construct mixtures of the effluents of source categories for ambient air mixtures to include inorganic vapor toxicants to complex PM exposures (Campen et al. 2013). The toxicants contained additional concentration increments of criteria pollutant vapors, i.e., either SO₂ or NO₂ (nitrogen dioxide). They also exposed the animals to SO₂ and NO₂ alone. They found that most of the cumulative responses in their ApoE^{-/-} mice that were found with the original complex mixtures were potentiated by the incremental exposures to NO₂, demonstrating that the additional vapor increment either changed the

chemical content of the mixture, or that the NO₂ affected the epithelium in a way that made it more susceptible to the effects of the original mixture.

Background on Inhalation Exposures to Complex Ambient Air Mixtures that vary Temporally and Spatially

In anticipation that the effects of inhalation of ambient air complex mixtures would be greater than the sum of the effects of its constituent components, or even synergistic, Chen et al. (2013), in the NYU NPACT study supported by HEI, exposed their ApoE^{-/-} mice to PM_{2.5} CAPs (concentrated 8 to 10-fold) at five different sites (New York City, Tuxedo, NY, Lansing, MI, Seattle, WA, and Irvine, CA). These sites varied in the concentrations and relative contributions of the sources to the PM_{2.5}, with the overall concentrations varying from site-to-site, being much lower at each site than those in the Campen et al. (2013) LRR study, and having greater differences in the concentrations of components attributable to major sources. The PM_{2.5} CAPs were produced using the versatile aerosol concentration enrichment system (VACES) described above. Identical systems were set up for use in Sterling Forest (SF) in Tuxedo, NY, at Mount Sinai School of Medicine (MS) in Manhattan, in Seattle, WA (SEA), East Lansing, MI (EL), and in Irvine, CA (IR). The mouse exposure, the monitoring of the exposure atmosphere and ambient aerosol particles, and the exposure concentration calculation were performed as previously described. The filtered air (FA) controls were exposed using an identical protocol, with the exception of a high-efficiency particulate-air (HEPA) filter positioned in the inlet valve position to remove all of the CAPs in the FA sham exposure stream. During the non-exposure period, mice were kept in the animal facilities with HEPA-filtered air at all locations. In this study, they found that the effects of the exposure on acute cardiac function were most closely associated with the residual oil combustion source, while the growth of aortic plaque during the 6 months of exposure was most closely associated with the coal combustion source.

Delivery of Controlled Exposure Atmospheres

The controlled exposure atmospheres need to be delivered to the breathing zones of the test subjects without undergoing any appreciable physical or chemical change in transit. The exposures can take place:

- within a whole-body exposure chamber holding multiple human or animal subjects;
- within a smaller head-only exposure chamber accommodating multiple subjects;
- within face masks;
- via a mouthpiece; or
- via nasal catheters

In addition, *in-vivo* exposures to PM can take place via intra-tracheal (IT) instillation, intranasal instillation, and/or by oral pharyngeal aspiration as a means of delivering well-defined doses to specific respiratory tract anatomical regions, and for exploratory studies using rare and/or materials that are difficult to resuspend.

Each of these delivery options has advantages and limitations.

Intranasal (IN) instillation exposure method (for mice and rats)

For each intranasal instillation, mice were anesthetized in a closed container containing Isoflurane (1–3% in O₂) (Butler Schein, Dublin, OH). After an animal was fully anesthetized, it was placed in a supine position on an inclined plastic platform, and administered a volume of approximately 5–50 µl of a solution or a suspension, one drop at a time, unilaterally to the right/ left nares/ or bilaterally with a micropipette. Animals were monitored for recovery immediately after IN administration.

Intratracheal Instillation (for mice and rats)

Animals were anesthetized as described above and placed in a supine position on an inclined plastic platform. A 24-gauge needle attached to a 1-ml syringe was inserted into the trachea via a modified otoscope. An appropriate concentration of a solution or suspension in a volume of 200 µl for rat or 50 µl for mouse was administered into the trachea. For mice, it may be necessary to expose the trachea via a small incision on the ventral neck skin. For rats, the oropharyngeal cavity is sufficiently large to visualize the epiglottis, and the needle can be inserted into the trachea without making any incision. The wound was closed with sterile wound clip.

Oropharyngeal Aspiration (OPA, for mice only)

Animals were anesthetized, as described above, and placed in a supine position on an inclined plastic platform. The tongue was gently pulled out of the mouth using forceps to visualize the base of the tongue and the pharynx. By timing the pharyngeal reflex, an aliquot of the treatment solution or suspension (50µl) was pipetted onto the back of the tongue at the beginning of the gag reflex, which allowed the liquid to be aspirated into the lungs. The tongue was released after at least two breaths had been completed. Because rats do not exhibit pharyngeal reflex under anesthesia, this technique was generally not used in this species.

Whole-Body Exposure Chambers

Subjects in whole body exposure chambers, both humans and animals, have minimal stress, and can engage in physical activities to enhance minute volumes and thereby simulate outdoor activity or occupational tasks, but require quite large volumes of the exposure atmospheres. The animal subjects may collect the test agent on their fur, and ingest it during grooming.

Head-Only Exposure Chambers

Subjects exposed in head-only exposure chambers, typically rodents, need elastic sleeves around their necks, and are much more physically confined during the exposures, and require physical handling, which can be stressful. An advantage is that the only exposure to the test agent is via inhalation.

Face Masks

Human subjects can be exposed via a whole face or half-face mask and require a minimal volume of the test agent.

Mouthpieces

Human subjects can be exposed via a mouthpiece and requires only a minimal volume of the test agent. They can also avoid deposition of the test agent in the nares and nasal passages, thereby normalizing the dosage delivered to the lungs.

Nasal Catheters

The use of nasal catheters to bypass the upper respiratory tract also requires only a minimal volume of the test agent, but is less acceptable than a mouthpiece to human subjects. It is an effective means of delivering test aerosols to the lungs of laboratory animals that are obligatory nose breathers.

When using masks, mouthpieces, and/or catheters for delivering aerosols to the lungs, it is important that the carrier stream be at body temperature and water vapor saturation, since bypassing part or all of the upper respiratory tract can lead to drying of the epithelium of the larger lung airways.

Design and Operation of Inhalation Exposure Facilities

While both human volunteers and laboratory animals undergo inhalation exposure studies in enclosures that are designated as exposure chambers, the chamber designs, support facilities, and operating procedures are usually quite different. Perhaps the biggest differences for human and animal exposure chambers are: 1) the space required per test subject, and the amenities, with humans being exposed in much larger spaces; and 2) the number of subjects undergoing the test, with only one or a few human subjects being studied at a time, typically for periods of a few hours or less. By contrast, animal inhalation chambers need to be capable of providing uniform exposures over many hours for large numbers of test subjects, while providing individual access to drinking water and management of excreta.

Human Exposure Chambers

These are typically functional hospital rooms that contain the equipment to be used for physiological assays, such as spirometry, diffusing capacity, and cardiac function, as well as the collection of blood samples, excreta, sputum, and sometimes nasal and lung-lavage samples. Some of the basic elements of a system for the exposures of human volunteers to ambient air pollutants were described by Bell et al. (1980) (See Figure 4).

Animal Exposure Chambers

While brief exploratory studies of a small number of animals can be conducted in static chambers or small chambers with recirculated air, as described by Cheng and Moss (1985), this review will be limited to dynamic flow chamber systems having constant delivery of planned concentrations of a test agent or mixture for the preselected exposure interval. There must be continuous air quality monitoring of the test agent in the breathing zone of a test

subject or a reference subject that can be shown to have an exposure that differs little from that received by others in the same test chamber. The continuous monitoring can be for a specific test agent, or at least a reliable surrogate index for the agent or mixture, with means of adjusting the input of the test agent(s) or carrier air in order to maintain the air temperature, humidity, and exposure target concentration(s). For mixtures, the constancy of the concentration ratios need to be verified by the collection and laboratory analyses of integrated air samples of the mixed exposure samples.

A key factor in our ability to deliver the exposure atmosphere with the desired characteristics to the breathing zones of the test subjects is our capacity to control the flow patterns of the test atmosphere within the exposure chamber. There should be as direct a path from the chamber inlet to the individual breathing zones as is possible, and with as little contamination as possible of the stream from air contaminant sources within the chamber, such as body effluents, re-suspended surface dust and lint, and, in animal chambers, from bedding, feces, and ammonia from decomposing excreta. Another complication in animal exposure chambers with large numbers of animals is the partial blockage of the flow stream by the support systems that hold the cages in place and by the trays below the cages that catch the excreta. The challenge in designing animal inhalation chambers is to ensure that the exposure atmosphere surrounding each cage is reasonably uniform for each animal. The best way to document that the composition and concentration of the test agent is sufficiently uniform within the chamber is to collect and analyze samples collected at multiple locations within the chamber, and to modify the positions of the cages and or support surfaces as necessary to obtain sufficiently uniform concentration distribution. A minimum of 10 air changes per hour would ensure sufficiently uniform distribution of the test material. As a further precaution, some investigators put only one animal in each cage to prevent them burying their nose in the fur of an adjacent animal in the same cage as a filter to minimize the concentration that they actually inhale.

There are two major design approaches for whole-body inhalation chambers for large numbers of small animals. These are vertical flow and horizontal flow. As described by Drew (1978), the classic vertical flow chambers were pioneered, by Laskin and colleagues, using a hexangular cross-section for exposures to uranium compounds at the University of Rochester as part of the Manhattan Project during World War II, by Stokinger and colleagues using square cross-sections (Fraser et al. 1959) at the Public Health Service's Occupational Health Program's laboratory in Cincinnati, OH, and by Laskin and colleagues at New York (Drew 1978). In this type of chamber, the exposure atmosphere is introduced at the top into a tapered transition zone that leads into the exposure zone in the middle. There is another tapered zone, below the exposure zone, that leads to the air exhaust pipe (see Figure 5A for the hexangular cross-section chambers at the University of Rochester, and Figure 5B for the square cross-section chambers at New York University). A variant in this design, developed by Moss (1978) to improve the uniformity of exposure within the chamber, has been used by the inhalation toxicology programs at: the Battelle Pacific Northwest Laboratory at Hanford, WA; Lovelace Respiratory Research Institute (LRRI) in Albuquerque, NM; and the Chemical Industry Institute of Toxicology (CIIT) in Research Triangle Park, NC, is illustrated in Figure 5C. The other main design approach (Figure 5D)

utilizing horizontal flow, was developed by Ferin and Leach (1980) at the University of Rochester, and is utilized at the Fraunhofer Institute in Germany (Hollander et al. 1988).

Nose-Only Exposure Chambers

One disadvantage of a whole-body inhalation exposure for laboratory animals is that test agent will deposit on the fur. While the material deposited on the fur is unlikely to have any direct effect on the animal, it may be ingested when the animal grooms itself, and end up in internal organs. This pathway for uptake can be avoided by exposing the animals using nose-only inhalation chambers, as illustrated in Figure 6.

Nose-only exposures have other advantages as well. These include:

- The exposure is limited to the test agent, i.e., there is no exposure from other sources in the chamber.
- The volumetric flow into the exposure chamber is much smaller than that in a whole-body chamber, which may be advantageous when the amount of the test agent is limited, or it is very expensive.
- There will be less variation in inhaled volumes, since all of the animals will be sedentary during the exposures.
- There will be less solid waste for disposal and cumbersome cages, trays, and interior chamber walls to wash between exposures and less waste on the exhaust air filters used to clean up the effluents from the exposure chambers.

The major downsides of using nose-only exposure chambers are:

- the greater amount of technician time required in loading and recovering the animals for each exposure session.
- the greater stress on the animals associated with their greater confinement.

Face Masks, Mouthpieces, and Nasal Catheters

There are other options for exploratory and special investigations involving inhalation exposures for limited numbers of both human volunteer subjects and experimental animals. They can be performed when suitable chambers are not available, and can be done with very small amounts of the test agents.

We have used face-masks for nasal inhalation exposures of submicron sized sulfuric acid droplets for studies of its effects on the mucociliary clearance of monodisperse inert particles in humans (Leikauf et al. 1981, 1984, Spektor et al. 1989) in rabbits (Chen and Schlesinger 1983), and for studies of the deposition and nasal airways clearance patterns of monodisperse inert particles (Lippmann 1970). We have used mouthpieces for the oral inhalation of monodisperse inert particles from the tracheobronchial airways of human volunteer subjects (Lippmann and Albert 1969, Chan and Lippmann 1980), and we have used nasal catheters to expose the lungs of donkeys to fresh cigarette smoke to study its effects on mucociliary clearance of monodisperse inert particles (Frances et al. 1970).

Characterization of Exposure Atmospheres

Temperature and humidity of inhalation chambers should be monitored continuously. Mass concentrations can be determined gravimetrically in a temperature and humidity controlled weighing room with a microbalance. The compositions of CAPs can be determined by X-ray fluorescence (XRF). Particle size distribution can be measured using scanning mobility particle sizing (SMPS). We have performed strong acidity analysis as well as soluble ion concentrations ($\text{SO}_4^{=}$, NH_4^+ , and NO_3^-). Details of these measurement methods have been described previously (Maciejczyk and Chen 2005). Control animals need to be exposed to an identical protocol, with the exception of a HEPA filter positioned in the inlet valve position to remove all of $\text{PM}_{2.5}$ in the filtered air stream. During the times between exposures, the animals are kept in housing facilities with filtered air.

Evaluation of Biological Responses to Inhalation Exposures - Respiratory Function (invasive and non-invasive)

There are many excellent and comprehensive reviews in the literature on the purposes and techniques for the measurement of respiratory function and its responses to inhaled toxicants, such as Mauderly (1995), which emphasized those used at the Lovelace Respiratory Research Institute (LRRI) in Albuquerque, NM, and Costa et al. (1991), which emphasizes those used at EPA's inhalation toxicology laboratory in Research Triangle Park, NC. As noted by Mauderly (1995), such techniques "provide information on the presence (whether or not function is impaired), nature (type of impairment), and extent (magnitude of impairment) of function loss. Sequential changes in pulmonary function measured serially provide information on the stage and progression of the underlying lung disease." However, changes in function induced by bronchospasm can be transient and unrelated to long-term structural changes, can be localized rather than spatially homogeneous, and be not responsive to epithelial derangements that can lead, over time, to pathological changes. Thus, respiratory function changes are a useful supplement to, and not a substitute for, histopathological evaluations.

Commonly encountered approaches for the measurement of respiratory function include:

- analyses of successive sample volumes of exhaled gas
- analyses of inspiratory and/or expiratory flow rates
- plethysmography, i.e., analyses of displaced volumes in a chamber that encloses the thorax
- alteration in breathing patterns, lung flows, and compliance
- flows and volumes during a forced exhalation
- washout of a bolus of an inert gas
- alveolar-capillary gas exchange

The following provides brief descriptions of methods used to measure respiratory function in rodents at NYU.

Forced pulmonary maneuvers (Buxco)

Mice are anesthetized with an intraperitoneal (IP) injection of ketamine (130 mg/kg) and xylazine (8.5 mg/kg) to maintain spontaneous breathing under anesthesia. Mice are tracheotomized, placed in a body plethysmograph and connected to a computer-controlled ventilator (forced pulmonary maneuver system, Buxco). Three different maneuvers are performed semi-automatically according to instrument instructions to obtain Boyle's Law FRC, TLC, VC, IC by quasi-static pressure-volume maneuver, forced expiratory flows (PEF and FEF), times of expiration and inspiration (T_e , T_i) and FEV₁₀₀ and FEV₂₀₀ by a fast flow volume maneuver (Carey et al. 2007; Farraj et al. 2006; Farraj et al. 2010; Vanoirbeek et al. 2010).

Diffusion capacity (DLco)

As done previously in our laboratory (Amdur and Chen 1989; Chen et al. 1987; Chen et al. 1990), after lung volumes are measured using the Buxco system described above, apnea is induced by hyperventilation. The lungs are then inflated with a volume of test gas (0.3% neon (Ne), 0.3% CO) equivalent to the inspiratory capacity (IC). Lung inflation is maintained for 6 seconds, and the Ne and CO concentrations in the end expiratory samples are analyzed on a gas chromatograph (Inficon 3000 Micro GC). DLco and the apparent alveolar volume (VA) are calculated by standard formulae. The averages of two measurements of DLco are used.

Forced oscillation technique (FlexiVent)

After the forced body plethysmographic measures, mice are transferred to a Flexivent system (SCIREQ, Montreal) to measure: 1) total respiratory resistance (R) and elastance (E) during oscillations ($V_T=10\text{ml/kg}$; $F=150\text{ breaths/min}$, $PEEP=2.0\text{ cm H}_2\text{O}$); and 2) Newtonian resistance (R_n , a measure of central airways resistance), tissue damping (G, a measure of resistance in the peripheral airways and parenchyma), and tissue elastance (H, a measure of elastance in the peripheral airways and parenchyma) (Carey et al. 2007; Vanoirbeek et al. 2010). Baseline values for each mouse are obtained by applying a 2 s perturbation at a frequency of 2.5 Hz followed by an 8 s pseudo-random perturbation consisting of waveforms of mutually prime frequencies (0.5 to 19.6 Hz) a total of 3 times at 30 s intervals.

Airway hyperresponsiveness (FlexiVent)

Mice are exposed to increasing concentrations of aerosolized methacholine (Mch) while in the Mch in doubling concentrations (4 to 64 mg Mch/ml) are nebulized through an inlet of the chamber. The response to saline or Mch is measured over the aerosolization period (1 min), an aerosol drying step (2 min), and then an additional 1-, 2-, 3-, 4-, 8-, or 12-min period (after exposure to 0, 4, 8, 16, 32, or 64 mg Mch/ml). After subtracting baseline values from responses to saline, the area under the curve (AUC) R, E, R_n , G and H are calculated using a trapezoidal method (Gavett et al. 2003). Changes of AUC for these parameters are indicative of alterations in airway responsiveness to Mch challenge.

Biochemical, Cellular, Histopathological, and Morphologic Measurements

The following provides brief descriptions of methods used pre- and post-exposure to measure biochemical, cellular, histopathological, and morphologic effects on inhalation exposures in rodents at NYU.

Lung lavage

Animals are euthanized with 175 mg/kg pentobarbital, IP. A tracheal cannula is inserted, the right lung is tied off at the right mainstem bronchi with suture material, and the left lung lavaged 2 times with 37°C PBS at a constant pressure of 25 cm H₂O and a volume equal to approximately 80% of TLC for that lung. One aliquot of the pooled lavage is analyzed for total cell counts (hemocytometer) and cytocentrifuged (Shandon) for differential counts. Lavage fluid is examined for general markers of pulmonary injury (protein and lactate dehydrogenase), as well as for cytokines (IL-1, 6, 10, 13, MCP-I, MIP-1, TNF α , using ELISA). Lung tissue is homogenized for determination of thiobarbituric acid-reactive (TBAR) substance (a marker of lipid peroxidation) content and the antioxidants, nonprotein sulfhydryl (90% glutathione) and ascorbate.

Histopathology

The slip-knot at the mainstem bronchi is released and the right lung is instilled, via the airways, with 2% glutaraldehyde in 0.1M cacodylic acid buffer at 25 cm H₂O. After 10 min, the lungs are removed, placed into fixative, and stored at 4°C. After 72 hr in fixative, the total volume of each lung is measured using the volume displacement method of Scherle. Lung volumes are compared to those measured with the Ne dilution method (separate groups of animals performed with pulmonary function measurement) to ensure that lung inflation is similar for all animals. Each lung is then divided for processing according to the following system: a section from the right lower lobe is removed and processed for embedding in paraffin, after which 5 μ m sections are cut from each block, placed on slides, and stained with hematoxylin and eosin for immediate screening of animal health; 2 mm sections at 1/3 and 2/3 the distance from the top of the left lower lobe are taken and processed for embedding in glycomethacrylate (PolyScience, Warrington, PA), after which a 3 μ m section is cut from each block and stained with Lee's methylene blue-Navy eosin. Pulmonary lesions and morphometric analysis are performed according to the method described previously (Kimmel et al. 1997).

Nasal Lavage (in mice)

The optimal procedure for trans-pharyngeal nasal lavage in mice is as follows: 1) Place fully anesthetized mouse with the ventral side facing outward and give an IP injection of pentobarbital (120 mg/kg). Wait & check to see if the mouse is down by applying pressure to the foot or tail with forceps; 2) Exsanguinate mouse; 3) Remove head and mandible as pictured in Figure 5A. If the mandible is not removed prior to a trans-tracheal lavage, PBS will leak out of the mouth resulting in loss of sample as well as sample contamination from the oral cavity; 4) Draw 0.5 ml of sterile PBS into the syringe, and then attach a clean cannula (rinse the cannula 3 times between each animal); 5) Gently perform a dual flush of

the nasal cavity from the posterior opening of the nose with 0.5mL of PBS (using the same 0.5ml); 6) Record the total amount retrieved, place lavage fluid into its own tube, and place on ice immediately; and 7) Separate cells from the supernatant by centrifugation, and place in refrigerator. Freeze supernatant for protein analysis. Do not freeze any supernatant needed for LDH measurements.

Planning for the Examination of Responses in Organ Systems Beyond the Respiratory Tract

The respiratory tract may be the only, or the primary, health risks of some inhaled toxicants but, for other toxicants, or for some components of toxic mixtures that have ready access to the blood stream, the effects in other organ systems may pose equal or greater health risks. Some now classic examples of inhaled complex mixtures of particulate matter and vapors that have produced significant health risks in organs outside the respiratory tract include: combustion effluents such as cigarette smoke (Chen et al. 2010c) for cardiovascular disease and COPD; the effluents from the combustion of diesel fuel (Quan et al. 2010a) and lung cancer; the coal smoke source for ischemic heart disease and lung cancer mortality (Thurston 2013); and the residual oil combustion source for cardiovascular hospital admissions (Lippmann et al. 2006, 2013). Other pathways from deposition sites in the respiratory tract to critical target sites include the sinuses for nanoparticles that reach the brain (Oberdorster et al. 2002), and for very thin fibers that reach the pleural and peritoneal spaces and cause mesotheliomas (Lippmann 2014).

Exacerbation of CVD Following Inhalation Exposures

PM_{2.5} exposures result in progression of atherosclerosis, alterations in vasomotor tone and potentiation of vascular inflammation.

Vascular Studies in ApoE^{-/-} mice

The ascending aortas are removed, and thoracic aortic rings (2 mm) are suspended in individual organ chambers filled with PSS buffer (pH 7.4), aerated continuously with 5% CO₂ in O₂ at 37°C, as previously described (Sun, 2005, 2009). Briefly, for vasoconstrictor responses, vessels are allowed to equilibrate for at least 1 hour at a resting tension of 700 mg before being subjected to graded doses of serotonin (5-HT, 10⁻¹⁰ mol/L to 10⁻⁵ mol/L), or phenylephrine (PE, 10⁻⁹ mol/L to 10⁻⁵ mol/L). Responses are then expressed as a percentage of the peak response to 120 mmol/L of KCl. The vessels are then washed thoroughly and allowed to equilibrate for 1 hour before beginning experiments with acetylcholine (ACh). After a stable contraction plateau is reached with 5-HT, which is about 50% of peak tension generated with maximal dose KCl, the rings are exposed to graded doses of the endothelium-dependent agonist ACh (10⁻¹⁰ mol/L to 10⁻⁵ mol/L).

Morphometric analysis of atherosclerosis in ApoE^{-/-} mice

Segments of aorta, brachiocephalic and left common carotid arteries are frozen in liquid nitrogen, and embedded in OCT compound (Tissue-Tek®, Sakura Finetek) for histopathological and immunohistochemical analysis (Chen 2009a, Quan 2009, Sun 2005).

Antibodies against CD68, iNOS, and eNOS are purchased from Santa Cruz Biotechnology. A polyclonal anti-nitrotyrosine antibody is obtained from Upstate Cell Signaling Solutions. Immunohistochemical staining is performed using the primary antibodies (1:200 concentration) and the Immunoperoxidase Secondary Detection System (Chemicon International), and quantified with software (NIH Image) after digitization of the images with Zeiss Axioskop/Spot 2 CCD camera system (Diagnostic Instruments) (Sun, 2005). At least 10 sections are stained per mouse, and quantification is done blindly. The data are expressed as the percentage of the lesion staining positive for the protein.

Circulating and Bone Marrow Endothelial Progenitor Cells

Both endothelial damage and repair are involved in the development of various types of cardiovascular disease (CVD), especially in early stages (Feletou and Vanhoutte 2006; Giannotti and Landmesser 2007; Jevon et al. 2008; Meyers and Gokce 2007). It is possible that ROS produced by CAPs promotes inflammation and induces deterioration in endothelial function in the vessel wall that either triggers or exacerbates the atherosclerotic process (Chevion et al. 2000; Stocker and Keaney 2004; Tahara et al. 2001). We have recently demonstrated, in ApoE^{-/-} mice, that long-term exposure to inhaled nickel hydroxide nanoparticles, a component of ambient PM_{2.5} (Chen et al. 2010b), induces oxidative stress and inflammation in both the lung and cardiovascular system, ultimately contributing to progression of atherosclerosis (Kang et al. 2010). Acute exposure to inhaled nickel nanoparticles significantly increased both bone marrow EPCs, which are endogenous semi-pluripotent stem cells that aid in endothelial repair, as well as their levels in circulation (CEPCs). Circulating endothelial cells (CECs), an index of endothelial injury, were significantly elevated indicating that endothelial damage occurred due to the exposure. There was no significant difference in EMPs between the two groups. Tube formation and chemotaxis, but not proliferation, of bone marrow EPCs was impaired in the nickel nanoparticle exposed group. These results coincided with a decrease in the mRNA of receptors involved in EPC mobilization and homing. These data provide new insight into how an acute inhalation nickel nanoparticle exposure may adversely affect EPCs and exacerbate cardiovascular disease states. Details of these assays were described previously (Liberda et al. 2010; Liberda et al. 2014).

Liver

For the liver endoplasmic reticulum stress and for oxidative stress, the experiments include gene expression analysis, immunohistochemistry staining, protein level analyses, and activity analyses. In addition, measurements of lipid profiles, regulation of lipogenesis, fatty acid oxidation, as well as glucose metabolism in the liver of the mice are measured. These include lipid and fatty acid profiling, glucose synthesis and usage (energy) analyses. Furthermore, hepatic inflammation and liver fibrosis should also be examined. These include hepatic inflammation scoring, signal transduction analysis, and liver fibrosis scoring.

An expert liver pathologist is needed to evaluate H & E stained sections for grading and staging of steatohepatitis in a blinded fashion using the modified Brunt scoring system (Tan et al. 2009). For quantitative analysis of collagen 1 staining by Sirius red, 90 images from

each liver are examined by Bioquant morphometric analysis (Safadi et al. 2005). Ten different low power images are taken from 9 separate sections from each liver. Macrophage infiltration is detected by CD68 staining, and activated stellate cells by α smooth muscle actin staining. The mean number of positively stained cells (for each cell type mentioned) in ten high power fields from three separate sections is calculated for each liver. Fat content, apoptosis, and oxidation increase in the early stages of non-alcoholic fatty liver disease (NAFLD). Therefore, Oil-Red-O (neutral fat content), TUNEL and cleaved caspase 3 (apoptosis), and hydroxynonenol (HNE) (oxidation) staining are performed on frozen sections.

Comparative quantitative real-time polymerase chain reaction (QRT-PCR) is performed to assess the cytokine milieu and stellate cell activation. Additionally, expression of selected other genes can be examined, such as PPAR γ 1, PPAR γ 2, PPARdelta, acetyl-CoA carboxylase (ACC 1, ACC2), carnitine palmitoyltransferase 1a, and p450 enzymes (CYP2E1, and CYP1A1), which may regulate NAFLD progression. Western blot studies of hepatic lysates subjected to SDS-PAGE are performed to compare QRT-PCR results with protein expression levels.

Making On-Line Noninvasive Functional Assays During and Between Inhalation Exposures

Telemetry for the Evaluation of the Effects and Interactions of PM Exposures on Heart Rate (HR) and Heart Rate variability (HRV)

Baseline ECG tracings and HR are monitored continuously for 1 week before CAPs exposures, and continue throughout the duration of a study, including during the exposure period as described previously (Chen et al. 2010b). ECG parameters (HR, body temperature, and activity) are monitored for 30 seconds at 5-minute intervals, 7 days a week, for the entire exposure regimen.

The times in milliseconds (ms) of occurrence of two consecutive R waves in ECG channel (RR) is calculated on a beat-to-beat basis using the computer package of Dataquest A.R.T. Analysis software (DataSciences). Only normal-to-normal (NN) intervals between 100 and 200 ms with NN ratios between 0.8 and 1.2 are included for analysis. Two time-domain HRV indices are measured: SDNN, the standard deviations of the RR intervals, and RMSSD, the square root of the mean squared differences of successive RR intervals. Frequency domain indices include high frequency domain (HF) and low frequency domain (LF). The LF/HF ratio is also measured as a frequency domain outcome that reflects the balance behavior of the sympathetic vs. parasympathetic components of the nervous system.

For each physiological outcome of HR and HRV parameters, the daily averages of each mouse at assigned time periods were calculated separately, based on the recorded 5-min RR intervals within each hour. For each, the mean of the daily average outcome of an animal was calculated and treated as the outcome baseline at the time period for the mouse. For each mouse, the outcome baseline was subtracted from all the daily average outcomes at the same time period. These daily baseline subtracted outcomes, called daily outcomes for short hereafter, were the data for statistical modeling.

Detailed statistical methods to measure acute and chronic effects on mice's HR and HRV changes were described in detail previously (Chen et al. 2010b). Briefly, we used our recently developed non-parametric method (Chen et al. 2010b) to estimate the time and magnitude that mean HR, HRV, body temperature, and physical activity differed significantly between various emission particles and sham exposed groups. These response variables were used in a two-stage modeling approach to estimate chronic and acute effects on the changes of these three response variables. In the first stage, a time-varying model was used to estimate daily crude effects. In the second stage, the true mean of the estimated crude effects was modeled with a polynomial function of time for chronic effects, an index function of emission particles for acute effects, and a random component for unknown noise. A Bayesian framework was used to combine these two stages to estimate the chronic effects of emission particles on HR and HRV.

Making Serial Non-Invasive Structural Assays Between Inhalation Exposures with Ultrasound Biomicroscopy

Atherosclerosis and cardiac function measurements

We have used an ultrasound biomicroscopy system (UBM, Vevo 770, VisualSonics, Toronto, Ontario) to measure the progression of atherosclerosis and cardiac function after combustion emission particle exposures as described previously (Chen et al. 2010d; Quan et al. 2010b). The single-crystal mechanical transducer has a central frequency of 40 MHz, a focal length of 6 mm and a frame rate of 30 Hz. The maximum field of view of 2D imaging is 10×10 mm with spatial resolution of ~60 µm (lateral) by ~30 µm (axial). The Doppler pulse repetition frequency is up to 96 kHz, corresponding to a maximum unaliased velocity of 120 cm/s (with incidence angle being zero).

Animals are anesthetized using 1.5% isoflurane and laid supine on a platform. Body temperature is maintained at 36–38°C using a circulating warm water heated pad. In each mouse, cardiac imaging (both B and M-modes) are obtained. We also measured the following parameters: diastolic and systolic left ventricle inside diameter (LVID, both B and M-mode), diastolic and systolic ventricle posterior wall thickness (LVPW, M-mode), diastolic and systolic interventricular septum thickness (IVS, M-mode), left ventricle outflow tract diameter (LVOT, B-mode), and right ventricle outflow tract diameter (RVOT, B-mode). From these measurements, left ventricle mass (LVM), left ventricle stroke volume (LVS), left ventricle ejection fraction (LVEF), left ventricle percent fractional shortening (LVFS), and left ventricle cardiac output (LVCO) were calculated.

We obtained the following parameters: peak velocities of E and A waves of mitral inflow, left ventricle isovolume relaxation time (IVRT), left ventricle isovolume contraction time (IVCT), aortic maximum velocity (Ao Vmax), pulmonary artery maximum velocity (PA Vmax), and maximum velocity for smaller innominate and carotid arteries. These measurements were used to detect stenosis of blood flow at these areas.

To measure the progression of atherosclerosis, we focused on the plaque lesions in the brachiocephalic and left common carotid arteries. In ApoE^{-/-} mice, the atherosclerotic plaques often occur in aortic sinuses, along the lesser curvature of the aortic arch, and in

innominate artery and the proximal part of common carotid arteries (Rosenfeld et al. 2000). The morphology (size and shape, e.g. intima-media thickness), distribution, of the atherosclerotic plaques in these arteries expresses many features of the human plaque that are very relevant to the pathogenesis of clinically significant disease (Getz 2000; Rosenfeld et al. 2000). Ten B-Mode movies (in cross-sectional position) were obtained along the brachiocephalic and left common carotid arteries at approximately 333 μ m gaps. From each of the second, third, and fourth movies, three pictures were based on the following criteria: pictures were clear and representative of the entire movie; and all pictures selected were electrocardiogram (ECG)-synchronized. For each picture, plaque area was measured using NIH Image and freehand drawing, and expressed as percentage of plaque area relative to the cross-sectional vessel cavity area. For each artery, the percent area in each of three locations was then averaged, and expressed as the percent volume of the plaque in a 1 mm length of each artery.

Reproductive Studies

Mating and Gestation

Mice 10–12 wk of age are used for mating. To obtain timed pregnant mice for each set of studies, a single CD1 male mouse is paired with two females (considered GD 0). After overnight pairing, the females are checked for vaginal plugs. Those showing no evidence of a plug are paired a 2nd time and inspected again after 24 hr. On GD 1 (24 hr after plug visualization), females (two per cage) are exposed for 4 hr/d (via nose-only inhalation) to either “fresh” or “aged”/agglomerated/calcined selected metal nanoparticles (NP) or to size-matched carbon particles/filtered air (as controls) until giving birth, or until the end of exposure on GD 17 (and sacrifice on GD 18).

Biologic Parameters

Duration of gestation is determined as previously described (Ng et al 2006). Pup birth weight is determined from PND 1–21 by weighing each pup from at least 10 different litters (within a given treatment group) on an analytical balance.

Mice have a built-in “indicator” of approaching parturition, namely development of a long ligament (ILL) between pubic bones accompanied by increased flexibility of the pelvic girdle; without these changes in flexibility, parturition may fail. These ligaments grow in preparation for parturition under the stimulus of increasing levels of estradiol and relaxin. This process also depends on a decrease in serum P that acts to antagonize the effects of estradiol and relaxin on the pubic symphyseal connective tissue as delivery approaches. Growth of the ILL is determined in dams from dams sacrificed at GD 18. The ILL of NP-exposed mice will be longer (compared to those of the agglomerated particles or carbon controls), indicating that the hormonal changes leading to parturition were accelerated. To measure ILL, the pubic symphysis is exposed by dissection (as in our previous studies with cigarette smoke and measured using a dissecting microscope fitted with an ocular micrometer and a trans-illuminating device (Ng et al 2006).

Summary and Conclusions

Toxicological testing by the inhalation route is much more complex and expensive than by other routes of dose delivery. When multiple subjects are exposed, the delivered doses to the respiratory tract and to other internal organ systems are more variable, since they depend on the respired volumes and flow rates, the various designs and operational features of the exposure chambers, and the chemical and physical characteristics of the administered chemicals, especially when particles of varying particle sizes are involved. The cost differentials between inhalation testing and dosing by other routes multiply when chronic effects are to be studied. On the other hand, the associations of occupational and/or environmental exposure concentrations and durations and biological responses are much more realistic for dose administration by inhalation when the results are to be used in health-related risk assessments. The advantages of inhalation studies for risk assessment are greatest when the exposure involves complex mixtures of gases, vapors, and particles of various sizes and aqueous solubilities, such as ambient air aerosols and smoke from cigarettes, stationary source combustors, and motor vehicles.

In this chapter, we review the historical backgrounds of developments in inhalation toxicology methodology, protocols, and facilities for dose delivery to the respiratory tract, the design and operation of human and animal exposure chambers and means for exposures of individual subjects, the characterization of the exposures, and the evaluation of biological responses to inhalation exposures in the respiratory tract and more distal organ systems (cardiovascular, hepatic, and reproductive). We also emphasized that, because of the complexity and high costs of inhalation studies, that great care should be taken in the design and execution of such studies so that the scientific returns can be maximized both initially, and in followup utilization of archived samples of the exposure atmospheres, excreta, and tissues collected for histology.

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Section 2. NPACT animal toxicologic study of cardiovascular effects of mixed vehicle emissions combined with inorganic particulate matter. Research Report 178

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Figure 1.
Schematic diagram of the furnace system to produce combustion emission source.

Figure 2.
String Generator Setup modified from Ledbetter et al (1998).

Figure 3.
Schematic diagram of the VACES system for CAPs exposure and telemetry monitoring (Maciejczyk et al 2005).

Figure 4.
Schematic of components of a system for inhalation exposures of human volunteers to a simulated ambient air pollution mixture. (from: Bell et al. 1980)

A) Schematic drawing of the Rochester Chamber
 B) Schematic drawing of the New York University Chamber
 C) Schematic of multitiered chamber showing the airflow pattern and designation of the different tiers
 D) Horizontal flow unit under test at the University of Rochester

Figure 5.

A. Schematic diagram of the University of Rochester whole body chamber. (from: Drew 1978).

B. Schematic diagram of the New York University whole body chamber. (from Drew 1978).:

C. Schematic diagram of the Moss whole body chamber. (from: Griffis et al. 1981)

D. Ferin and Leach (1980) horizontal-flow whole body chamber. (from: Ferin 1978).

A

TOP VIEW

Top view of head-only chamber.

B

Flow-Past Chamber. Aerosol enters at chamber INLET, filling inlet manifold (1) and flowing directly through the horizontal branch (2) to the open end of the animal container (3). Unused aerosol and exhaled air returns directly to the exhaust manifold (4), then to the chamber EXHAUST.

Figure 6.

A. Schematic diagram of a nose-only inhalation chamber: Top View. (from: Drew et al. 1980).

B. Flow pathways for rodent inhalation and exhalation in a nose-only exposure system. (from: Cannon et al. 1981).