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The
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Development

Pollution and Heart Disease

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The Contribution of Air Pollution to the Burden of Cardiovascular Disease

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The first people to experience the health effects of air pollution were undoubtedly the first people to use fire for cooking and staying warm. However, it wasn't until people began to live together in cities and began to burn coal and other fossil fuels, for industrial processes and transportation as well as food and warmth, that medical professions began to seriously consider the adverse health effects of these combustion products. Now that our society and economy are dependent on the benefits of burning these fuels, we live in an atmosphere that is made up, not only of "natural" gases (such as nitrogen, oxygen, carbon dioxide) and aerosols (such as wind-borne dust), but also potentially harmful gases, such as ozone, aldehydes, and sulfur dioxide, and aerosols consisting of microscopic droplets of such chemicals as sulfates and nitrates and carbon particles ("soot") to which chemicals such as hydrocarbons and heavy metals are adsorbed. This is especially true in large metropolitan and industrial areas. As a result of this increase in air pollution, the biomedical community began, especially in the last century, to investigate the health effects of air pollution on the large number of people who are now exposed.

For obvious reasons, most research was initially focused on the toxic effects of air pollution on the lungs, and it was shown that air pollution can exacerbate chronic lung diseases, including asthma, in adults and children. However, in the early and middle 1990s researchers began to find, somewhat surprisingly, that air pollution also appeared to be associated with mortality from cardiovascular disease (CVD). Two landmark studies in this area – the Six Cities Study and the American Cancer Society Study – that looked at large populations of people exposed to a wide range of levels of pollution strongly suggested that cardiovascular disease was associated especially with the particulate component (often referred to as "particulate matter" or "PM") of air pollution (1,2). Since those initial studies, many reports have confirmed a robust link between exposure to PM and cardiovascular disease and death, and more recent reports have also suggested a connection between ozone exposure and CVD. Publications in leading cardiovascular journals and other high impact journals, including a Scientific Statement on "Air Pollution and Cardiovascular Disease by the American Heart Association (3), suggest that the cardiovascular community is recognizing the potential contribution of environmental exposure to risk for CVD.

The significance of an air pollution–CVD connection could be quite profound. While the associations that have been seen are fairly small, often less than 1% increase in risk per 10 microgram/cubic milliliter difference in PM levels, many people are exposed to air pollution, so the cumulative effect could be great. In addition, cardiovascular disease (sometimes referred to simply as "heart disease") is the primary cause of death in the industrialized world. According to the American Heart Association, 927,448 people died of CVD in the United States in 2002. This represents a death rate of 320.5 per 100,000 people and accounts for 38.0% of all deaths (4). Depending on how significant a risk factor air pollution exposure proves to be, relative to the other known risk factors – age, race, lifestyle factors (such as smoking, physical inactivity, and diet), serum lipids, and family history – reduction in air pollution levels could be a means for significantly reducing the burden of CVD.

However, epidemiologic associations, although they may be strong, don't completely make the case. There is also the issue of "biological plausibility": does the association make sense based on what we know, or what we can learn, about the how cardiovascular diseases develop? In other words, are there pathologic mechanisms to explain the association? This represents a major focus of research in the area of pollution-related CVD.

Several mechanisms to explain air pollution toxicity have been proposed and all have data to support them. These ideas developed mostly from research on the effects of PM. The most obvious explanation is direct toxicity to the cardiovascular tissue. This seems most plausible for chemicals that can be solubilized in the lung after inhalation, such as aldehydes and metals dissolved from particulate matter, and transported to the cardiovascular tissues, most likely through the blood. However, there is also evidence that the particles can be translocated intact from the lung to vascular and cardiac tissues. This is especially true for the

smaller, so-called, ultrafine particles. Once in the vicinity of the CV tissue, the pollutants might adversely affect the tissue by causing or increasing local inflammation, interfering with metabolic processes, disrupting ion channel function, etc. The pollutants might also cause local inflammatory responses in the lung that result in production of inflammatory mediators that could be transported to the cardiovascular tissues, thus affecting function. Either of these two mechanisms might explain experimental data showing an increase in atherosclerosis in animals exposed to PM (5).

A third hypothesis is that pollutants might trigger nerve endings in the lung which subsequently send impulses to the brain and in turn back to the heart. These nerves are part of the autonomic nervous system and influence how the heart beats. This has been offered as a mechanism to explain evidence that PM in the lung can affect the rhythm of the heart. It is of course likely that all of these mechanisms play a role.

There is one more important question that research needs to answer: Why are individuals affected differently by exposure to air pollution? What are the individual "susceptibility factors" that make one person more at risk from exposure to air pollution? Evidence has begun to suggest that the elderly and those with pre-existing disease are most affected. It is also reasonable to suspect that air pollution might worsen the effects of the "traditional" risk factors for CVD, such as smoking and diet. And finally, an active area of research in all areas of disease susceptibility is the role of genetics. A coordinated approach between basic laboratory science and population-based science is needed to fully resolve this question.

In the articles that follow, researchers from the University of Louisville, who are active this area of research, discuss many of the important considerations involved in trying to understand air pollution-related cardiovascular disease. First, Drs. Prough, Amunom, and Conklin provide an overview of the chemistry of some chemicals in air pollution, such as polycyclic aromatic hydrocarbons and aldehydes. In addition, they outline the metabolic processes the body uses to try to eliminate these exogenous chemicals, and importantly, how the processes sometime create metabolites that are more toxic than the original compound. Finally, they discuss some of the consequences of the chemical reactions of these compounds with DNA and proteins. In another chapter, Pierce and coworkers describe the use of mass spectrometry in defining protein adducts and their contribution to cardiotoxicology.

Drs. Hill, Barski, and Bhatnagar then review some of the epidemiologic findings linking air pollution and cardiovascular disease, as well as data linking occupational exposures to cardiovascular disease. They also discuss work in their lab and in others' that is looking at how exposure to aldehydes can cause alterations in serum lipid profiles, a known risk factor for cardiovascular disease in humans, and can reduce the body's ability to protect the heart against ischemia, a common cause of heart attacks. Dr. Prabhu's overview focuses more on the effects of particulate matter, for which there is the most data related to cardiovascular disease. In addition, he briefly discusses some of his research on aldehydes which shows that acrolein, a common aldehyde in various kinds of smoke including tobacco smoke, depresses the heart's ability to function optimally, perhaps due to its effect on the protein that causes the heart muscle to contract.

Research on the health effects of air pollution continues to be an active and crucial scientific effort. New epidemiologic data strengthen the case for a link between exposure and cardiovascular disease, as well as other conditions not previously considered, such as adverse birth outcomes. As these data accumulate, the need to understand how these pollutants cause their effects becomes more important as well. Agencies such as the National Institute of Environmental Health Sciences (NIEHS) and EPA are currently funding research, such as that described here at the University of Louisville, to try to elucidate these mechanisms. The results of this research cannot only lead to a reduction in the rate of disease from air pollution exposure through enactment of appropriate air pollution standards, but can also add to our overall understanding of the causes and the pathologic mechanisms of heart disease.

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The Role of Metabolism in Protection Against Cardiotoxic Compounds

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Abstract

Combustion of organic matter in the presence of oxygen leads to complete combustion forming CO_2, water, and oxides. Incomplete combustion leads for pyrolysis forming either fused ring molecules or unsaturated compounds, like acrolein and formaldehyde. The unsaturated compounds are chemically reactive and directly alter cell growth and differentiation. The fused ring compounds are chemically stable, unless metabolized by enzymes in our bodies to reactive intermediates. We have evolved several families of enzymes which detoxify these reactive intermediates. This article will review how products of pollution are formed, how they directly or indirectly react with biomolecules, and how they are toxic in the heart and other vascular tissues.

Introduction

The concern that many products of combustion can cause adverse biological effects in mammals has stimulated research that provides a partial understanding of how compounds, like benzo[a]pyrene (B[a]P), cause specific cancers, such as small cell carcinoma. Like many other environmental chemicals, B[a]P, in and of itself, is not chemically reactive, but must be metabolically activated by enzymes in our cells. In the case of B[a]P, a polycyclic aromatic hydrocarbon [PAH], it has been well documented that it is formed during the combustion of tobacco or other organic materials and has the potential to cause cancer in animals and man. An elegant set of experiments performed in the 1970s (Holder *et al*., 1974; Selkirk *et al*., 1974; Kapitulnick *et al*., 1977; Levin *et al*., 1986; Chang *et al*., 1987) documented that benzo[a]pyrene requires metabolism in mammalian tissues to form the ultimate carcinogenic agent. This reactive intermediate reacts with specific DNA bases in target genes to form DNA-PAH chemical adducts, thereby causing the critical somatic cell mutations that lead to cancer. Many of these studies were performed in animal models and a series of biological oxidative reactions studied in these models were shown to form the 9,10-epoxy-7,8-dihydrodiol-benzo[a]pyrene, the ultimate carcinogenic form of B[a]P (Holder *et al*., 1974; Levin *et al*., 1986). The realization that such reactive intermediates are formed in our bodies with our own enzymes has been a paradox to understanding the carcinogenicity and/or toxicity of such compounds, since normally these enzyme systems detoxify foreign compounds by converting them to water-soluble, easily excreted metabolites. This article will review some of the compounds formed during the combustion process or inflammatory processes and how they are detoxified on the one hand and cause environmental disease on the other.

Compounds formed during the combustion process.

During the combustion processes associated with open fires, burning tobacco products, and automobile fuel combustion, organic materials are heated to extremely high temperatures and sometimes at high pressures in the presence of oxygen. Under conditions of complete combustion, *i.e*., in the presence of excess oxygen, most organic materials are converted to carbon dioxide and water, while compounds containing heteroatoms such as sulfur and nitrogen, are converted to ammonia, SO_2, nitrogen oxides, *etc*. Such combustion reactions are known as complete combustion reactions and normally produce new chemical products with low chemical reactivity.

The energy associated with this process in the absence of excess oxygen causes pyrolysis reactions that lead to condensation reactions and formation of PAHs and decomposition reactions to small, reactive organic molecules, such as carbon monoxide (CO), acrolein and formaldehyde. This process is known as incomplete combustion and often forms chemicals with higher chemical reactivity. In addition, toxic metal oxides are produced from the metals in the organic material, such as arsenic used as wood preservatives, lead in older paints, etc. Due to the intense temperature and diverse conditions created during burning, both degradative oxidation to compounds like carbon dioxide and water are formed (expected during complete combustion). During

incomplete combustion, unburned organics are formed including fused ring compounds, such as the polynuclear aromatic hydrocarbons, such as naphthalene, benzo[a]pyrene, and chrysene or carbon monoxide, aldehydes like acrolein and formaldehyde, sulfur dioxide, and nitrogen oxides produced (expected during incomplete or partial combustion). In the case of the smaller compounds, such as CO, sulfuric acid, hydrochloric acid, hydrofluoric acid, nitric acids and NO derivatives, these compounds can react with biological molecules, like hemoglobin, cytochromes, glycolytic enzymes, and alter their function; while the polynuclear hydrocarbons, dioxins, and furans may be largely chemically inert under these same conditions. Due to the diverse products formed from fire or other smoke producing processes, molecules are generated that either act directly to alter biological molecules or subsequently become metabolically activated to reactive intermediates by the enzymes in our body.

Electrophilicity and adduct formation: The chemical nature of toxic organic materials.

The foreign compounds formed by combustion processes can cause a number of responses. First, these compounds cause direct chemical or toxicological reactions of the electrophilic compounds formed during combustion. An electrophilic agent is one which is electron deficient, such as αβ unsaturated aldehydes (formaldehyde and acrolein), sulfur dioxide or nitrogen oxides. These compounds react with various cellular nucleophiles (electron-rich compounds), like glutathione [g-glutamyl-cysteinyl-glycine], a tripeptide thiol capable of reacting with reactive electrophilic compounds which like to gain electrons into their structure (Figure 1). Thus, other biological nucleophiles, like proteins or nucleic acids, can also form such adducts with electrophilic

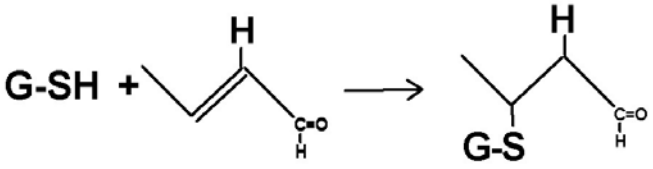

Figure 1. The reaction of the cellular nucleophile, glutathione or -glutamyl-cysteinyl-glycine, with the combustion product acrolein (2-propenal) to form the GSH-acrolein conjugate.

compounds. The aldehydes described above are examples of reactive electrophiles formed during the combustion process that are directly reactive with biological materials like proteins and DNA or the cellular nucleophiles.

These reactive chemicals are produced by the combustion process; however, new reactive intermediates may be

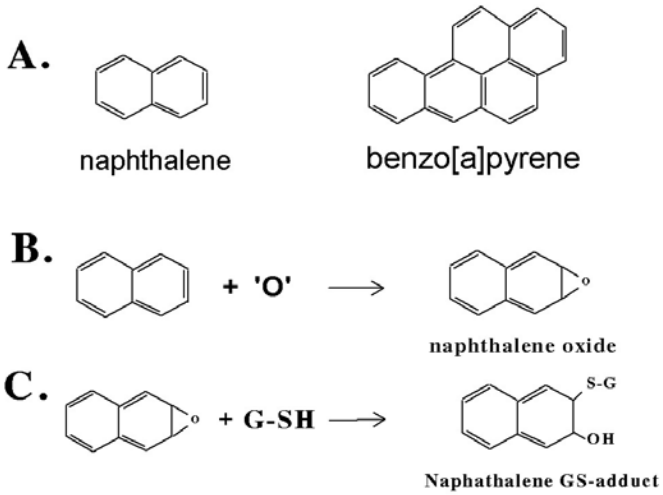

Figure 2. The metabolism of polycyclic aromatic hydrocarbons. A. Naphthalene and benzo[a]pyrene. B. The oxidation of naphthalene to form naphthalene oxide. C. The reaction of glutathione with naphthalene oxide to form the glutathione conjugate of naphthalene.

formed biologically when enzymes in our bodies catalyze modification of various functional groups on other compounds formed during combustion. For example, combustion causes formation of PAHs during incomplete combustion of organic materials, including cigarette smoke. Examples of these compounds would include naphthalene and benzo[a]pyrene (Figure 2A). In and of themselves, these molecules are not chemically reactive, but upon metabolism by oxidative enzymes known as monooxygenases, these PAH can be converted into epoxides, compounds which are also electrophilic in nature (Figure 2B). Like the αβ unsaturated aldehydes, epoxides are capable of reacting with cellular nucleophiles, such as glutathione, proteins and nucleic acids of DNA (Figure 2C). So in summary, combustion produces compounds that are either direct acting chemicals, like αβ unsaturated aldehydes, or that require subsequent metabolic activation to produce reactive chemicals, like epoxides of polynuclear aromatic compounds.

The Body's Defense: Mechanisms of foreign compound elimination.

Many of the organic compounds produced by combustion are fat-soluble and are taken up by our fatty tissues as a storage depot. This is one reason that exposure of mammals to foreign compounds is hazardous; i.e., their lipid-solubility causes the compounds to accumulate in the body and be stored for long periods of time. An example of this situation is the storage of polycyclic aromatic hydrocarbons and compounds like dioxin (formed both by forest fires and by man) in the fatty tissues of our bodies after exposure or ingestion of these materials. Without methods to remove these compounds from the environment so they won't accumulate

TABLE 1. Common enzymes involved in foreign compound metabolism

Enzyme	Reaction type	Reactant	Cofactor
Cytochrome P450	HydroxylaseEpoxidase	Molecular oxygen	Reduced pyridine nucleotides
Glutathone S-transferase	Thiol conjugation	Electrophile	Glutathione
Sulfotransferase	Sulfate conjugation	Phenol	3'-Phosphoadenosine 5'-phosphate
Glycosyltransferase	Glucuronidation	Phenol	UDP-glucuronic acid
N-acetyltransferase	Acetylation	Amines	Acetyl-Coenzyme A
Epoxide hydratase	Hydration	Epoxides	Water

in the body, we have to rely on a series of enzymes that aid in the elimination of these compounds from the body. The difference in the rate of accumulation of these compounds in our fatty tissues and their elimination by metabolism to more polar compounds dictates how fast they leave our body. One way to describe the duration of retention of compounds in our body is the term half-life, or the time it takes for Ω of the body burden of a compound to be eliminated through the kidneys or gastrointestinal (GI) tract.

Using a battery of enzymes that are oxidative, reductive and/or conjugative (i.e., linking a water-soluble compound to a fat-soluble compound) in reaction type, our organs, especially the liver, can metabolize both direct and indirect acting reactive chemicals by attaching an oxygenated functional group to the molecule. Subsequently, other enzymes add a water-soluble compound like a sugar, sulfate, water, or glutathione to the molecules. In the pharmacology literature, the oxidative/reductive enzymes have been termed Phase I metabolic enzymes; the conjugative enzymes are therefore termed Phase II synthetic enzymes. Finally, there are processes that extrude these compounds or their metabolites from the tissues into the blood stream, urinary tract, or GI tract for disposition from the body; this process is called transporter enzymes or Phase III enzymes. For most chemicals, these reactions produce compounds which are not toxic and by adding water-soluble molecules to these oxygenated molecules, are made more water soluble. This increased solubility allows these water-soluble materials to be removed from fatty tissues and organelles in our liver and other tissues and export them to the kidney or feces for elimination. If the compounds formed have little or no toxicity, then the compounds are rapidly and efficiently removed from our body. These processes decrease the half-life of organic molecules in our body through enhanced disposition after metabolism. Our livers are designed to be very active in eliminating such compounds from the body and serve as a "garbage system" for elimination of fat-soluble compounds, both foreign compounds and endogenous compounds, including cholesterol and fat.

The enzyme systems of importance that are involved in eliminating such compounds are listed in Table 1 above. The major oxidative enzyme encompasses a heme-thiolate protein family containing over 100 distinct genes in mammals. It carries out oxidation of carbon compounds by attaching an oxygenated functional group that eventually can become linked to water-soluble biomolecules, increasing the water solubility of these combustion products. The sorts of water-soluble molecules that are involved in conjugation reactions include glutathione (-glutamyl-cysteinyl-glycine or GSH utilizing glutathione S-transferases), glucuronic acid (UDP-glucuronic acid utilizing UDP-glycosyltransferases), sulfate (3'-phosphoadenosine 5'-phosphate utilizing sulfotransferase), acetate (acetyl-CoA utilizing N-acetyltransferases), and water (H_2O utilizing epoxide hydratase).

Many of the metabolic processes in mammalian cells have the ability to alter the expression of various genes to compensate for times when more or less metabolism is needed. These same processes exist in toxicology, since it has been shown that an organism is highly sensitive to a chemically-reactive material upon first exposure, but during subsequent exposure to the compound less toxicity results. This process is known as "tolerance" and can be explained by a number of biological mechanisms. One part of this process is known as "induction" and generally is due to an increase in transcriptional activity of gene expression and the accumulation of the protein catalysts in our tissues and cells. This inductive process allows compounds to which we are exposed to have the ability to increase their own metabolism; through activating specific receptors in target tissues like liver, these receptors function after chemical exposure, like the sex steroids act through nuclear receptors or transcription factors (Nebert *et al.*, 2004; Whitlock *et al.*, 1997). One particular receptor is the Arylhydrocarbon receptor (AHR) which is ubiquitously distributed throughout the body (Figure 3). The PAHs formed during incomplete combustion activate this receptor in those tissues that become exposed to these materials. Upon entry into the cell and binding to the AHR, the compounds cause the AHR to migrate

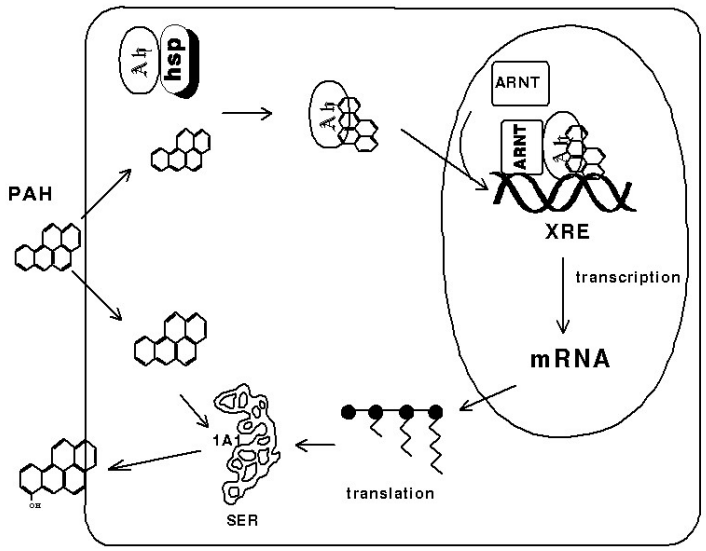

Figure 3. The arylhydrocarbon receptor and its action. Polycyclic aromatic hydrocarbons can passively diffuse into cells and interact with the Arylhydrocarbon receptor (AHR) in the cytoplasm. Activation of the protein complex associated with the AHR causes a migration to the nucleus where it combines with the arylhydrocarbon receptor nuclear transporter (ARNT) and bind to cognate responsive elements on the 5-flanking region of genes. This leads to the production of mRNA's capable of encoding enzymes which detoxify compounds like benzo[a]pyrene to hydroxylated metabolites that are not toxic. These metabolites must also be easily excreted from the body to diminish environmental disease.

to the nucleus of these target cells and activate a battery of genes involved in disposition of combustion products and other chemicals, eventually resulting in the increased expression of the messenger RNA and protein encoding the enzymes involved in foreign compound metabolism, such as cytochrome P4501A1, UDP-glucuronosyltransferase, *etc*. The enhanced excretion of the foreign compounds results from this increased expression of the genes involved in disposition of foreign compounds and enhances elimination of these compounds from the body. These receptors often also increase other biomolecules serving as protective agents for the body, like glutathione. The oxidative enzymes also catalyze a limited number of reactions leading to deleterious reactions, such as DNA and protein activation. The paradoxical dichotomy of these enzymes' action in activating a limited number of chemicals and their major role in eliminating most other chemicals, including those we take as drugs and beneficial compounds, causes us to be cognizant of the importance of limiting our exposure to combustion compounds and other materials that may be bioreactive or can be made bioreactive.

Many other ligand-activated nuclear receptors or transcription factors exist which can interact with foreign compounds as well as endogenous compounds. Another important receptor is the NF-E2-related (Rushmore *et al*., 1990; McMahon *et al*., 2991; McMahon *et al*., 2003) nuclear transcription factor (also known as Nrf2) which is released from a cytoplasmic protein complex upon binding of electrophilic compounds. The subsequent accumulation of Nrf2 in the nucleus activates another discrete set of target genes important for increasing the concentration of other enzymes, including GSH synthase, heme oxygenase, and many other protective enzymes. This regulatory system is involved in protecting against the direct acting chemicals formed like NOx, αβunsaturated aldehydes, sulfur dioxide, metals; i.e, the compounds also produced by the incomplete combustion process.

Reactive chemicals cause abnormal cell growth and proliferation.

In the past, considerable research has been directed toward understanding how toxic chemicals cause cancer. The consensus is that foreign compounds are converted to reactive electrophiles that can chemically react with cellular nucleophiles like protein and nucleic acid to form products known as adducts. Such reactions are believed to lead to toxicity by damaging proteins by forming protein adducts or carcinogenesis by forming DNA adducts that result in somatic cell mutations and cancer. However, the literature offers a second role for toxic chemicals in causing abnormal cell proliferation and inflammation that results in formation of aberrant tissue growth and development, such as atherosclerosis. Chemicals such as the a,b-unsaturated aldehydes or reactive electrophilic intermediates of polycyclic aromatic hydrocarbons may be part of this second mechanism for causing disease in humans (Bhatnagar and Srivastava, 1992; Ramos *et al*., 1996; Moorthy *et al*., 2002). Aberrant proliferation of vascular smooth muscle cells has long been known to allow these cells to calcify and form plaque in arteries and aorta. This aberrant cell growth, accumulation of lipid and calcification eventually results in closure of vessels leading to and throughout the heart, causing cardiovascular disease and atherosclerosis. Therefore, these exposures to reactive chemical intermediates can not only cause cancer, but also other chronic diseases like atherosclerosis, heart disease and possibly other diseases like diabetes. This is schematized in Figure 4 showing that aldehydes, R-CH=O, like formaldehyde or acrolein formed by decomposition of volatile organic compounds cause toxic endpoints such as atherosclerosis, dyslipidemia (excess lipid in the bloodstream), and endothelium dysfunction (inadequate endothelial cell function). These lead to diseases like diabetes, obesity, hypertension, etc.

The research being pursued at the University of Louisville in our laboratories focuses on how these disease

states arise and how they can be prevented. Our particular interest is to see how oxidative and reductive metabolism may blunt these toxic events, protecting against aberrant cell proliferation. A number of enzymes are involved in these oxidation-reduction processes (Figure 4 and Table 2). Aldose reductase (AR) or the aldo-ketoreductase (AKR) enzyme families chemically reduce aldehydes to alcohols, that tend to be less toxic than the aldehyde compounds. In addition, aldehyde dehydrogenases use $NAD(P)^+$ to oxidize aldehydes to carboxylic acids. Both the alcohols and and carboxylic acids can be conjugated with water-soluble compounds that enhance excretion through the kidney or into the bile. Recently, we have noted that the hemoprotein family, cytochrome P450 (also know as CYPP450), can also oxidize and reduce aldehydes to carboxylic acids and alcohols as well (Amunom *et al.*, 2005). This enzyme system has high capacity to remove these foreign compounds and appears to serve as a secondary system to protect the body from aldehydes when the capacity of the reductases has been attained.

Societal problems due to our reliance on modern chemical and electrical conveniences.

As seen in the cover of this issue of *Sustain*, a paradox exists in our modern life-style. The freeway shown with vehicles moving rapidly through the hospital curve at Louisville is a great benefit to our freedom to travel and transport goods around our city at will. The electricity produced by coal-fired power plants or products like hot water, etc. to which we have become accustomed all require the use of combustion of petrochemicals to maintain our lifestyles. From the 1950s to the 1980s, we slowly realized that the pollution formed from release of combustion processes like the automobile engine or coal-fired power plants or chemicals from our chemical plants pose a threat to our health through a number of malignancies. With the advances in industrial technology, workers are increasingly protected from direct industrial exposure to such chemicals produced in West Louisville. However, the wonderful freeway systems and coal-fired power plants continue to expose us to pollution that

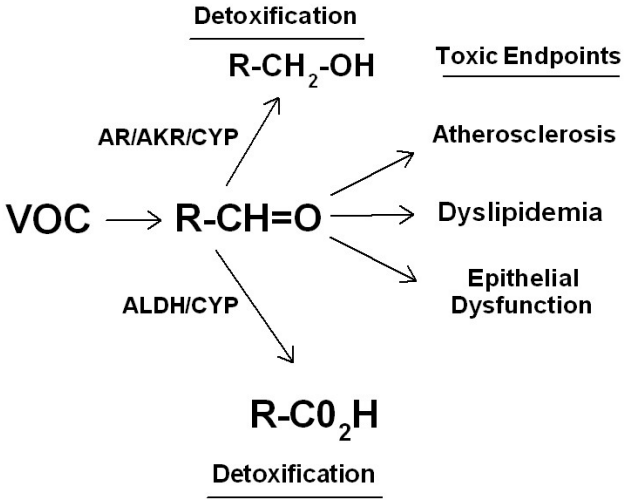

Figure 4. The role of metabolism in detoxification of aldehydes formed from volatile organic compounds that lead to aberrant cell proliferation. AR, Aldose reductase; AKR, aldo-ketoreductases; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; VOC, volatile organic chemicals; R-CH=O, chemical formula for an aldehyde.

appears to lead to deleterious diseases like atherosclerosis, dyslipidemia, and perhaps even diseases like diabetes. The coming decade will hopefully provide us with information about how the environmental pollution we produce in our modern lifestyle causes these emerging diseases threatening our populace. In some part, the knowledge we gain about how these processes work may allow us to prevent or treat these diseases and increase the quality and quantity of life for the Citizens of Louisville. However, this realization also forces us to devise ways to decrease further the chemical and particulate pollution we generate. Since all of these pollution products affect health and cause environmental disease, the other chapters in this issue will further document the role of such chemicals in emerging environmental diseases.

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Table 2. Enzyme systems involved in oxidative-reductive metabolism of aldehydes formed from volatile organic compounds

Enzyme	Reaction	Coenzyme	Reaction type
Aldose reductase	Glucose → Sorbital	NADPH	Reduction
Aldo-keto reductase	Aldehyde → Alcohol Ketone → alcohol	NAD(P)H	Reduction
Aldehyde dehydrogenase	Aldehyde → carboxylic acid	$NAD(P)^+$	Oxidation
Cytochrome P450	Aldehyde → carboxylic acid	$NADPH + O_2$	Oxidation

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ENVIRONMENTAL POLLUTION: RELATIONSHIP TO CARDIAC DYSFUNCTION AND HEART DISEASE

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Several pollutants are present in ambient air, and include particulate matter (PM) and gases. PM is a complex, dynamic mix of solid and liquid particles suspended in air. A large array of chemicals has been identified in PM. Some of the more common PM components include: 1) elemental and organic carbon, nitrates, and sulfates; 2) organic compounds such as aldehydes and polyaromatic hydrocarbons; 3) metals and metal oxides; and 4) biological compounds including bacterial products and pollen grains. Gaseous components include nitrogen oxides, sulfur dioxides, carbon monoxide, and ozone. PM is classified based on particle size; generally, an aerodynamic diameter < 10 microns (termed thoracic particles or PM_{10}) can penetrate and deposit in the lungs and are considered the most deleterious to health (Figure 1). According to current convention, $PM_{10\text{-}2.5}$ particles are referred to as the coarse fraction, $PM_{2.5}$ and less as fine particles, and $PM_{0.1}$ and less as ultrafine particles. Ultrafine particles have the potential for increased toxicity and may even pass directly into the bloodstream from the lungs.

Pollutants arise from both man-made and natural sources. Human-derived sources include industrial and fossil fuel combustion, motor vehicle emissions, and environmental tobacco smoke and are generally $PM_{2.5}$. Natural sources include windblown soil, forest fires, and bioaerosols and are generally $PM_{10\text{-}2.5}$. Ozone, a principal constituent of urban smog, is formed via the effects of ultraviolet radiation from the sun on both oxygen in the atmosphere and nitrogen oxides and hydrocarbons derived from human activity. It is important to recognize that PM composition is variable over time and location, and that the adverse health effects may be secondary to any one of the thousands of chemicals that have been detected in PM. Indeed, determining the precise effects referable to each constituent can be quite difficult. Furthermore, although PM refers specifically to air pollutants, many of its chemical constituents are also found in other environmental sources. One such example is a group of environmental pollutants termed aldehydes, which comprise an area of active research interest at the University of Louisville. While aldehydes are important volatile components of $PM_{2.5}$ and tobacco smoke, they are

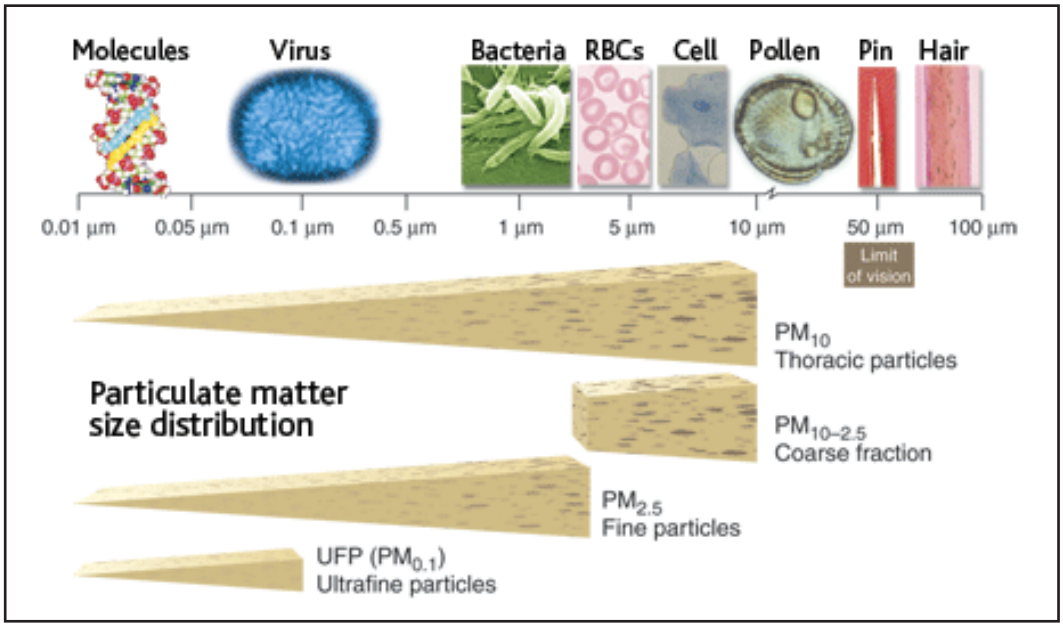

Figure 1. Classification of particulate matter. Adapted from Brook et al./ AHA Scientific Statement # 71-0289, URL: http://circ.ahajournals.org/cgi/content/full/109/21/2655/FIG1.

ubiquitous and are also present in water and food. Thus, the potential health effects of specific pollutants theoretically should consider exposure derived from all environmental sources.

The World Health Organization considers air pollution as one of the top 10 causes of disability that accounts for nearly 700,000 deaths annually. It is projected that if the current pattern of fossil-fuel consumption and emissions continues there will be nearly 8 million air pollution-related, and potentially avoidable, deaths by the year 2020. From a public health standpoint, while air pollution has classically been recognized as an exacerbating factor for lung diseases such as asthma and for the development of certain cancers, the epidemiological link between environmental pollution and heart disease has only been assiduously described in the last several years, and thus the concept of pollution as a risk factor for heart attacks, heart failure, and abnormal heart rhythms has been slower to gain acceptance.

The Jewish Hospital Heart and Lung Center located in the medical complex in downtown Louisville, KY.

Several population-based studies have demonstrated an important and potentially causative association between both short-term and long-term human exposure to PM and cardiovascular illness. These investigations have strong implications for the formulation of regulatory policies regarding acceptable levels of pollutants from human activity such as industrial and automobile emissions. Short-term studies have evaluated the relationship between recent pollution exposure and adverse outcomes. Although these are often analyses of heart disease in relation to the day-to-day variation in air pollution (generally 1-2 day lag period), the effects have been noted to persist when evaluated at longer periods after exposure (e.g., > 40 days). The initial impetus for such research was provided by the observation of higher mortality rates during extreme elevations in air pollution. During the London fog incident of December, 1952, for example, there was a precipitous increase in air pollution due to stagnant weather. Review of available hospital records from that time suggested an excess of 12,000 additional deaths over several months after the event due to both cardiac and respiratory causes.

More recent epidemiological studies conducted in the United States (National Mortality and Morbidity Air Pollution Study [NMMAPS]) and Europe (Air Pollution and Health: a European Approach [APHEA-2]) have shown remarkably consistent associations between deaths due to heart and lung diseases (i.e., cardiopulmonary mortality) and PM levels the day before death. The overall increase in risk (~0.2-0.5% for each increase in PM_{10} exposure of 10 micrograms/meter^3) is quite small compared to traditional risk factors for heart disease such as cholesterol levels, high blood pressure, diabetes, obesity, and cigarette smoking. However, since air pollution exposure is ever-present, the absolute number of people affected can be very large. For example, in one analysis of the effects of PM exposure in the Netherlands, a small European country, this 0.5% increase in risk translated into an excess of at least 2100 deaths per year, twice the number there due to traffic accidents. Direct associations have been demonstrated between PM and chest pain and heart attacks, heart failure and difficulty breathing, abnormal heart rhythms and shocks delivered from implanted defibrillators, and stroke. Recent studies have also indicated a relationship between spending time in traffic and the risk of a heart attack an hour afterward, and the level of ozone exposure and the number of heart attacks within a period of 1 to 2 days.

Of equal significance, studies of long-term exposure to PM have also demonstrated increased all-cause mortality, at a level of 4% for every 10 micrograms/meter^3 in annual $PM_{2.5}$. These investigations included the Harvard Six Cities Study results published in 1993 and the American Cancer Society Cancer Prevention Study published in 2004. Importantly, cardiovascular deaths, and not respiratory illnesses, were the single largest category of increased mortality due to chronic PM exposure, and were linked to ischemic heart disease (i.e., heart attacks and angina), abnormal heart rhythms, heart failure, and cardiac arrest. Studies on secondhand smoke (SHS), the largest contributor to indoor PM, underscore the chronic effects of PM exposure on cardiovascular health. Nonsmoking spouses of smokers have an estimated 25% increase risk of heart disease, and an estimated 2-fold increase risk of stroke. A more vivid example of the impact of SHS on heart disease was seen in June, 2002, when a public

smoking ban was instituted in Helena, Montana. In the first 6 months of the ban, hospital admissions for heart attacks dropped by 40%; this effect was not seen in hospitals from surrounding towns. The totality of evidence thus implies a causative link between ambient PM exposures and both acute and chronic cardiac illness. Additionally, several groups of individuals appear to be particularly vulnerable to these effects. These include the elderly, those with pre-existing heart and/or lung disease, those with diabetes, and those of lower socioeconomic status. Active tobacco smoking also increases the individual's susceptibility to heart failure, abnormal heart rhythms, and cardiac arrest due to pollution exposure.

Although the epidemiological evidence linking PM exposure and heart disease morbidity and mortality is strong, much less is known as to precisely how PM exposure engenders these effects. It is thought that fine air particles may impact our heart and blood vessels in several ways. They may have direct effects on the heart and arteries, as particulates such as ultrafine particles, bacterial products, and metals readily pass from the lungs after inhalation into the bloodstream. Fine particles can also initiate generalized inflammation in the body that can serve to constrict blood vessels and promote the formation of blood clots, and aggravate the progression of fat deposits in arteries. The right combination of abnormalities in susceptible persons can compromise blood flow in the arteries that supply the heart or brain, resulting in a heart attack or stroke. In this regard, it has been shown that short-term exposure to environmentally-relevant concentrations of a mix of $PM_{2.5}$ and ozone provokes arterial constriction and narrowing even in healthy adults.

PM inhalation can also alter the heart rhythm. Normally, the heart rate has a given degree of variability due to cyclic fluctuations in the output of the autonomic nerves to the heart that regulate the heartbeat. Exposure to ambient PM reduces this variability, perhaps related to the activation of neural reflexes in the lung in response to injury or inflammation, and the suppression of the normal output of the autonomic nerves. While in healthy individuals these effects may go unnoticed, in patients susceptible to heart arrhythmias significant abnormalities in the heart rhythm, and perhaps even cardiac arrest, can occur. This effect is particularly important in patients with heart failure, as such patients already have underlying abnormalities in the normal variability of the heart rate and heightened susceptibility to abnormal heart rhythms. Indeed, although death due to heart failure makes up about 10% of all cardiovascular deaths overall, heart failure deaths account for 30% of cardiovascular deaths related to PM exposure.

The development of strategies to reduce the cardiac health risk from pollution exposure requires public health and regulatory initiatives, as well as a scientific understanding of the underlying mechanisms responsible for these effects. From a population standpoint, more stringent regulation of environmental pollution is the most effective method to reduce adverse health effects associated with PM. This will require intense effort and collaboration between several groups including the public via their elected local, state and federal legislators, industry, and the scientific community. There also needs to be awareness of the potentially large costs involved for air pollution abatement, and consensus agreement as to what constitutes "acceptable" levels of exposure. However, discovering the underlying mechanisms is as important, especially for susceptible patient groups. What may be a tolerable level of exposure for healthy persons may produce adverse consequences in vulnerable individuals. At the University of Louisville School of Medicine, several researchers have been interested in determining the specific biological mechanisms that account for the association between environmental pollution and heart disease. As discussed above, the chemical makeup of PM is quite complex and a large knowledge gap exists regarding the specific health effects referable to select chemical constituents of PM. Due to several lines of epidemiological and experimental evidence, we have been particularly interested in a group of pollutants called reactive aldehydes. Our research group wants to determine how these pollutants affect the structure and function of the heart and the heart's ability to withstand reductions in blood flow, and how pre-existing heart disease (due to heart attack) changes the response to these pollutants.

We have an interest in aldehydes for several reasons. First, aldehydes are ubiquitous pollutants. They are generated during the combustion of fossils fuels, and are abundant in automobile exhaust, cigarette smoke, natural and man-made fires, and industrial waste, and are an important component of PM. Specific aldehydes such as acrolein have also been designated by the Environmental Protection Agency (EPA) as high priority pollutants in drinking water, and others such as hexenal are present in cooked foods and fats. Second, specific aldehydes (acrolein is one example) can be particularly toxic to biological systems. In the words of the EPA, "acrolein is estimated to pose the highest potential on a nationwide basis for significant chronic non-cancer effects." Third, exclusive of environmental exposure, all of us also generate small amounts of these reactive aldehydes in our own body during the course of normal metabolism – this balance can be radically altered in diseases such as heart failure. Such changes within the body may provide clues as to why certain

groups of individuals are more susceptible to environmental exposures of similar chemicals. Lastly, although several experimental and epidemiologic studies hint that aldehydes (derived either externally from the environment or internally within the body) contribute to heart dysfunction and cardiac disease, this relationship has not been systematically or critically examined and is poorly understood. Therefore, it is our intent to evaluate the consequences of aldehyde exposure on the heart and to probe the specific mechanisms by which this occurs.

Thus far, we have discovered several new and important effects of aldehydes on the heart. We have seen that acute exposure to acrolein, a model reactive aldehyde, can profoundly depress heart function and also increase the sensitivity of the heart to reductions in blood flow, or ischemia. These effects are reversible and may be related to selective effects of acrolein on the contractile proteins and energy-generating systems of the heart. These types of effects may be important during acute and/or occupational types of exposures and suggest that such aldehyde-induced effects can contribute to the reported increase in heart attacks and dysfunction upon short-term pollution exposure. Just as important, we have also determined that chronic oral exposure to acrolein induces inflammation in the heart resulting in the appearance of dilated and dysfunctional heart tissue suggesting incipient heart failure. These findings raise the intriguing possibility that environmental pollutants may contribute to the development of cardiomyopathy of otherwise undetermined origin. All of these studies underscore the potential interactions between pollutants (specifically aldehydes), heart dysfunction, and heart disease, and constitute the first experimental evidence suggesting that environmental aldehydes could trigger or affect clinical cardiac disease. In the next few years, we plan to do further experiments to expand on these early results, and develop a working model for how aldehyde pollutants contribute to and/or induce heart pathology.

In summary, the accumulating evidence supports the notion that environmental pollution is a novel and infrequently recognized risk factor for a variety of cardiac diseases including heart attacks, heart failure, abnormal heart rhythms, and sudden cardiac death. The mechanisms may be related to direct effects, indirect effects due to the induction of inflammation, and altered regulation of the heart rhythm. The importance of specific pollutants in producing adverse health effects is under active investigation; our work suggests an important role for reactive aldehydes in producing heart dysfunction, increased susceptibility to heart ischemia, and heart failure. A focused effort on the part of the public sector, industry, and researchers will be required to institute appropriate regulatory initiatives, and to combat the underlying mechanisms responsible for pollution-mediated heart disease.

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Getting to the heart of pollution: Is pollution a new risk factor for cardiovascular disease?

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Abstract

Pollution from the combustion of fossil fuels has emerged as a major contributor to environmental deterioration and human disease. Evidence accumulated over the last ten years supports an explicit link between heart disease and pollutant exposure, and it is currently believed that the majority of deaths due to air pollution are due to cardiopulmonary diseases. Thus, an understanding of how the constituents of pollutants in air, water, and cigarette smoke affect the cardiovascular system is of primary importance. Accordingly, rigorous studies within our laboratories are shedding new light on the mechanisms by which specific components of pollution contribute to heart disease.

Health effects of pollution are a growing concern worldwide. Ever since the industrial revolution, we have altered the environment in significant ways such that the results of our activities have proven to be harmful to wildlife, delicately balanced ecosystems, and our own health. We now clearly know that polluted air and water do not only affect rain forests and grasslands but also the risk, incidence, and severity of some of the most serious of human diseases. The extent of the problem or the degree to which pollution contributes to the overall burden of human disease is not known. It has been estimated that in 1997 alone there were 700,000 worldwide cases of avoidable deaths due to indoor and outdoor air pollution. These deaths have been linked to suspended particulate matter, largely from outdoor urban exposures. This does not include deaths due to other sources of pollution such as contaminated food or water. What is even more alarming is that the World Health Organization (WHO) estimates that by the year 2020 there will be eight million deaths related to air pollution alone (1). The situation is particularly grim in developing countries such as India where deaths due to pollution exceed those due to cancer and heart disease combined. The conditions in the developed world are not much better. Early work by environmentalists and concerned legislators has dramatically reduced the levels of several pollutants (lead, mercury, second-hand cigarette smoke), however, several toxic chemicals continue to pollute our environment and their harmful effects are only now coming to light. The most significant of these pollutants are the air-borne particulates. These are small invisible particles that are present in the air and are generated from human activity. Coarse particles are created by actions such as the grinding and grating of surfaces and the rubbing of tires on the surface of roads, whereas the finer ones emanate from automobile emissions and power plants (2;3).

A pivotal study linking air particulates to adverse health outcomes was published by a group of investigators from Harvard in which they showed that people living in cites with high levels of particulates had lower life expectancy than people living in more polluted urban areas that had subordinate levels of particulates. Their comparison was based on data collected from six cities across the United States and it showed that if you lived in Stubenville, Ohio you could expect to die earlier than if you lived in Topeka, Kansas (4). While the overall conclusion that pollution affects life expectancy was not a big surprise, the startling finding was the link between particulates and health outcomes. These particles are present in the air everywhere and are particularly high in urban areas. Does this mean that everyone is at risk, and, if so, to what extent and how does particulate air pollution contribute to chronic disease? The answers have been slow in coming and even after fifteen years of research and hundreds of published studies we have only marginally addressed these concerns. We know that particulate air pollution increases the incidence of cancer. Moreover, it can exacerbate asthma, and people with chronic lung disease are more susceptible to the harmful effects of air pollution. However, research since the famous six-city study has yielded another surprise. In analysis of the cause of deaths that could be linked to air pollution, it was found that most of the excessive deaths attributed to particulate air pollution were due to heart disease (5).

The reason that the link between heart disease and air pollution was surprising was that little was known about how environmental pollutants affect heart disease. Although there were a few studies linking heart disease and exposure to solvents, cardiovascular toxicity was not usually measured as an outcome of pollutant exposure, even

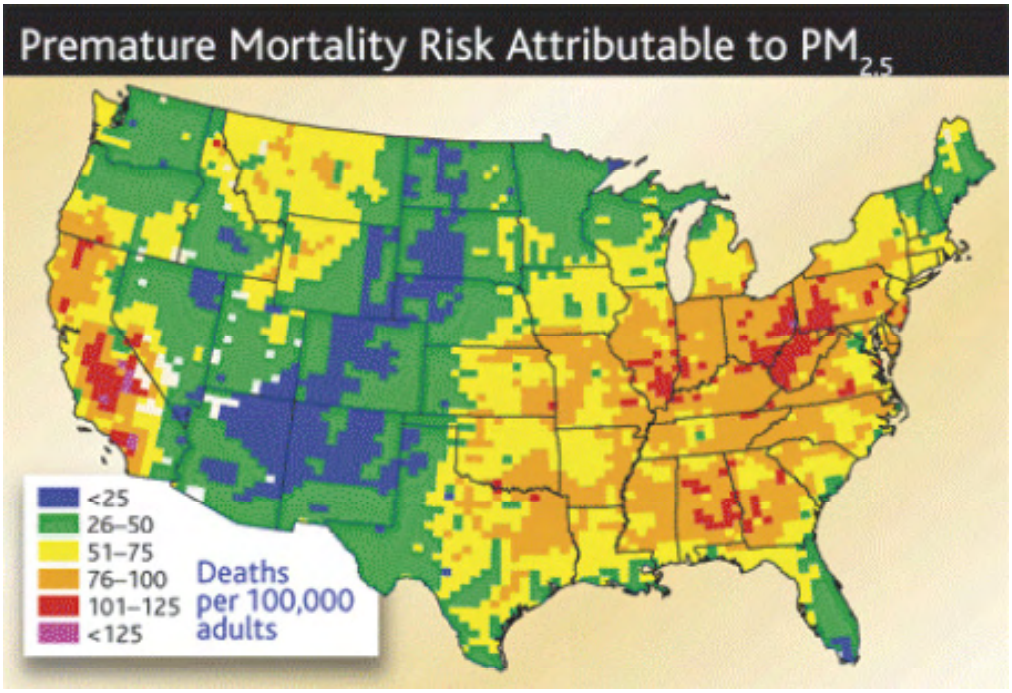

Fig. 2: Risk of premature death from PM2.5 particles in ambient air (from Science 307, 1858-1861, 2005).

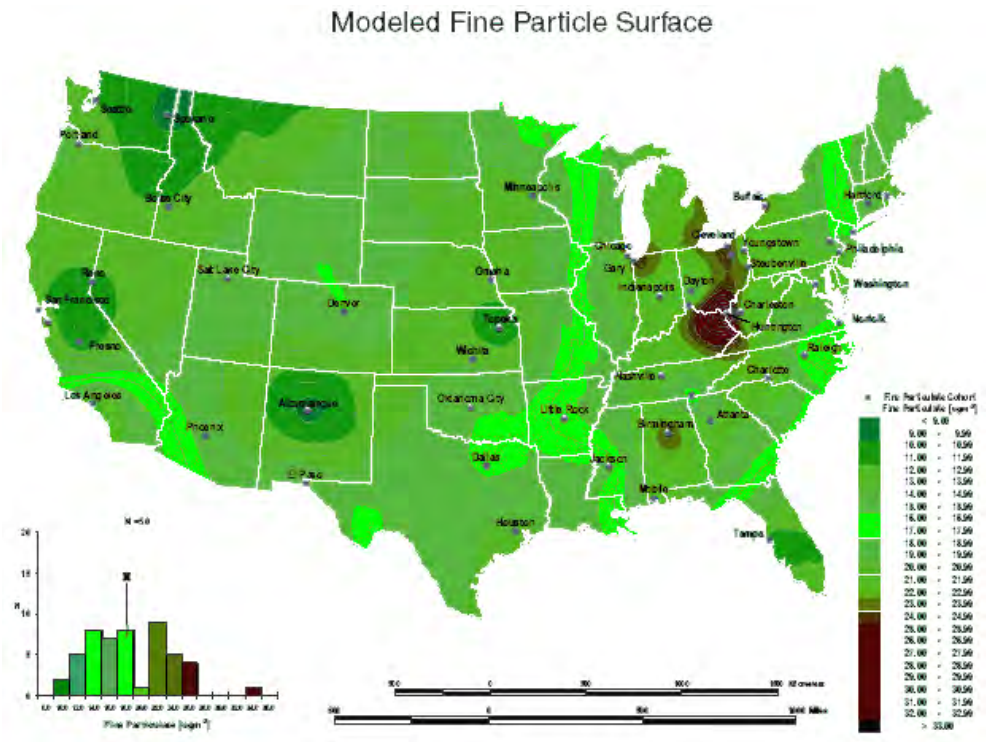

Fig. 1: Model PM_{10} distribution in the United States

in controlled laboratory conditions. The reasons for such omission are not clear but could relate to the tacit assumption that the heart and blood vessels were somehow protected from the environment. Overall, the emphasis was and is on cancer and lung disease because of the consequences pragmatic to air pollution. Thus, there seemed no reason to suspect that heart disease could be negatively affected by pollution. However decades of research on cigarette smoke, which is the most frequent toxic pollutant to which humans are exposed, indicate that quite the reverse may be true.

The unhealthy effects of cigarette smoke have been under scrutiny since the early 1950's, and we are only now beginning to recognize the complexity of mechanisms by which smoking contributes to several diseases. Nearly all of the initial studies focused on the relationship between cancer and smoking. However, several studies clearly show that smoking significantly affects heart disease, and heart disease deaths due to tobacco use far exceed the number of deaths due to lung cancer. In recent estimates, smoking kills five to six times more people by increasing heart disease than causing lung cancer (6). Fortunately, technology is now allowing researchers to identify thousands of compounds contained within cigarette smoke, and these investigations have already played a role in the realization that voluntary inhalation of cigarette smoke is detrimental to the cardiovascular system. More importantly, recent studies are revealing that sidestream (second-hand) smoke is drastically more toxic than mainstream smoke. Analyses in these investigations

demonstrated that sidestream smoke is approximately four times more toxic per gram than mainstream smoke, leading to the conclusion that "smoke-free public places and workplaces are the only practical way to protect the public from the toxins in sidestream smoke." Other studies have shown a direct link between second-hand smoke and cardiovascular disease, underscoring the evidence of smoking as the leading cause of preventable death (7). While this has been known for many years, it is only now that the full impact of these data is coming into view. The lessons learned from the health effects of smoking may be particularly useful as we begin to probe how other air pollutants affect heart disease. Cigarette smoke contains several pollutants present in ambient air (e.g., particulate matter, butadiene and aldehydes) that have been independently linked to heart disease (*vide infra*), suggesting that their presence could precipitate cardiovascular infirmities.

Overwhelming evidence accumulated over the last ten years supports the link between heart disease and air pollution (2;5;6). Over a hundred epidemiological studies have been published showing that deaths due to heart disease increase on days of high pollution. It has been shown that a sharp spike in air borne particles is followed one to three or twenty-four hours later by an increase in heart attacks. This relationship has been found in many cities as different from each other as Boston from London or New York from Los Angeles. Further analysis of the cause of mortality showed that most of the deaths were due to acute myocardial infarction (heart attack), however deaths due to arrhythmias and heart failure were also increased. The EPA estimates that of the 350,000 yearly cases of sudden cardiac deaths in the United States, 60,000 could be related to particulate air pollution (8). In general, it is currently believed that 70 to 80% of excessive deaths due to air pollution could be due to cardiopulmonary causes (9).

Therefore, if we accept the data that air pollutants and smoking increase the incidence of heart disease, then we are left with the question why? Again, no one knows for sure. Despite extensive epidemiological data published over three decades, it is still not known how smoking increases the incidence of heart disease. With particulate air pollution, the problem may be even more difficult to solve. The particles themselves vary greatly in size and composition and there is little agreement between the experts as to which components of the particles are causing the health effects. Thus, either the particles themselves or some other pollutant (e.g., sulfates, ozone, or nitrous oxides) that increases in parallel with particulate pollution could be causing the excessive mortality.

To simplify the problem and to study it rigorously within a laboratory setting, we have begun a series of studies on the aldehydic components of urban air and particulates. Aldehydes are reactive chemicals that are pervasive in our environment (10). They are present in food – the crisp smell of fresh fruit or spices is due to aromatic aldehydes such as citral (in citrus fruit) and cinnamaldehyde (in cinnamon) (Table 1-page 20). They are also responsible for the aroma of cooked food and the putrid smell of rancid meat (11). Aldehydes are also present in all types of smoke as they are inevitably generated by combustion (Table 2 and 3-page 21). One of the most common of these is acrolein – which is responsible for the acrid smell of smoke. Many aldehydes are also present in urban drinking water supply streams, as well as in streams, rain or fog. The EPA estimates that, with the exception of metals, aldehydes are the most prevalent pollutants in drinking water that represent the highest health threat.

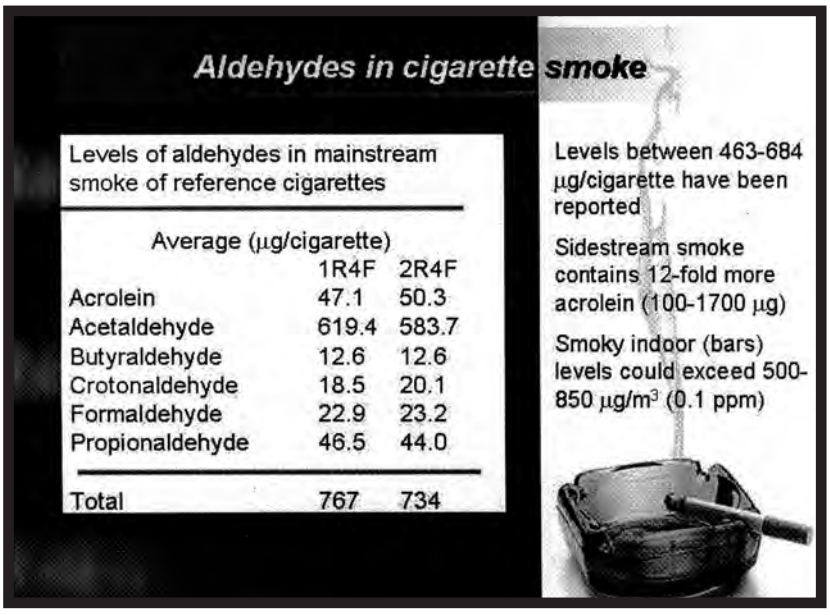

Figure 3

Not all aldehydes are bad, and several aldehydes such as those present in herbs, spices, and fruits have anti-inflammatory properties. Moreover, most of the aldehydes ingested are efficiently removed by the body by a variety of enzymes systems (e.g., aldehyde reductase, aldehyde dehydrogenases, alcohol dehydrogenases and glutathione-S-transferases) that have evolved specifically to detoxify foreign aldehydes. Thus, moderate aldehyde consumption or exposure does not elicit any untoward effects. However, persistent exposure to low levels of environmental aldehydes such as those generated in deep-fried food or cigarette smoke or those produced

TABLE 1

Aldehydes identified in foods*

Compound	*# foods in which the compound has been identified*	*Maximum concentration measured (ppm)*	*Food in which maximum concentration was measured*
Formaldehyde	40	98	Shellfish
Acetaldehyde	150	1,060	Vinegar
Malondialdehyde**	-	-	-
Butanal	60	51	Wheaten Bread
Acrolein	35	3.8	Red Wine
Crotonaldehyde	35	0.7	Red Wine
Cinnamaldehyde	30	815,000	Cassia (bark oil)
Furfural	150	255	Coffee
Glycidaldehyde**	-	-	-
Citral	15	130,000	Lime, peel oil
Anisaldehyde	10	25,000	Anise
Vanillin	30	23,200	Vanilla
Propanal	100	31	Wheaten Bread
2-methylpropanal	80	14	Whiskey
2-methylbutanal	40	2	Potato Chips
3-methylbutanal	80	73	Cocoa
Pentanal	100	14	Red Wine
2-pentenal	30	6	Heated butter
Hexanal	100	300	Orange, peel oil
2-hexenal	80	76	Banana
Heptanal	80	100	Orange, peel oil
2-heptenal	40	20	Orange, peel oil
2,4-heptadienal	50	15	Heated butter
Octanal	80	8,100	Grape, peel oil
2-octenal	30	100	Orange, peel oil
Nonanal	80	24,000	Orange, peel oil
2-nonenal	50	410	Kumquat, peel oil
2,4-nonadienal	30	1.5	Heated butter
Decanal	30	22,000	Orange, peel oil
2-decenal	30	200	Orange, peel oil
2,4-decadienal	80	500	Tangerine, peel oil
Undecanal	30	310	Lime, peel oil
Citronellal	25	2,000	Orange, peel oil
Benzaldehyde	150	3,000	Cinnamon
Phenylacetaldehyde	80	14	Tea, fermented

* Data in this table are mainly from Volatile Compounds in Foods (1989).
** Found in rancid foods

Table 1: Aldehydes identified in foods

TABLE 2

Composition of aldehydes present in automobile exhaust*

Aldehyde	Concentration (mole %)
Formaldehyde	36.5
Acetaldehyde	34.9
Propionaldehyde	3.6
Acrolein	1.1
Methacrolein	4.1
Benzaldehyde	11.3
Tolualdehyde	2.3
Ethylbenzaldehyde	3.2

*From Committe on Aldehydes: Formaldehyde and other aldehydes, Washington, D.C., Board on Toxicology and Environmental Health Hazards, Assembly of Life Sciences, National Research Council, National Academy Press, 1981, pp. 234-241

Table 2: Composition of aldehydes present in automobile exhaust

TABLE 3

Levels of aldehydes in mainstream smoke of reference cigarettes*

	Average (µg/cigarette)	
	1R4F**	2R4F**
Acrolein	47.1	50.3
Acetaldehyde	619.4	583.7
Butyraldehyde	12.6	12.6
Crotonaldehyde	18.5	20.1
Formaldehyde	22.9	23.2
Propionaldehyde	46.5	44.0

* From Dong JZ and Moldoveanu SC. Gas chromatography-mass spectrometry of carbonyl compounds in cigarette mainstream smoke after derivatization with 2,4-dinitrophenylhydrazine. *J. Chromatogr. A* 1027: 25-35, 2004.
** Reference cigarettes

Table 3: Levels of aldehydes in mainstream smoke of reference cigarettes

in the atmosphere from automobile exhaust and industrial emissions could be particularly harmful.

For now, it is not known for certain whether aldehydes in the atmosphere negatively impact human health and what those affects are. However, there are tell-tale signs or suggestions that exposure to aldehydes could be harmful, particularly to cardiovascular health. Many studies have shown that occupational exposure to aldehydes could increase the risk for cardiovascular disease. These studies, reporting an increased risk of atherosclerotic heart disease in embalmers (12), undertakers (13), perfumery workers (14), bus drivers (15), or workers in formaldehyde-producing plants (16), support the view that exposure to aldehyde-rich environments could be deleterious to cardiovascular health. Indeed, the recent push to ban smoking in public places such as restaurants and bars results from the realization that second-hand smoke is capable of precipitating acute cardiac events and may also negatively influence the chronic process of atherogenesis.

Exposure to industrial chemicals such as butadiene and vinyl chloride has also been linked to heart disease. In an extensive study of workers in styrene-butadiene rubber polymer manufacturing plants from 1943-1982, it was found that African-American workers were at a two-fold higher risk of developing heart disease than those who did not work at the plant (17). Interestingly, exposure to butadiene did not increase the risk for heart disease in Caucasian workers. These observations are of high local interest in Louisville and surrounding areas since there is a large rubber manufacturing plant in our community. As a result, urban air in areas of Louisville contains several times the EPA permissible limit of butadiene. While butadiene, which is also a component of cigarette smoke, is well known to cause cancer and trigger asthma, the realization that it could cause heart disease adds new interest in regulating its emissions and limiting exposure. The notion that chemicals such as butadiene could accelerate atherosclerosis is supported by animal studies which show an increase in atherosclerotic lesion formation upon exposure (18). Interestingly, these studies show that the amount of butadiene required to increase heart disease was much lower than that to cause cancer, suggesting that an increase in heart disease may be a more common or frequent outcome than cancer in people exposed to such chemicals. A similar increase in the risk for heart disease has been shown for workers exposed to vinyl chloride, which is another chemical used in the rubber industry. A seven-year study of 1,100 vinyl chloride exposed workers showed that exposure to vinyl chloride significantly increases the prevalence of high blood pressure and the incidence of heart attack (19).

While neither butadiene nor vinyl chloride is an aldehyde, we believe that the ultimate toxicity of these pollutants is related to their conversion to aldehydes. Both these compounds are metabolized by the body which converts them to aldehydes and these aldehydes are in turn responsible for the adverse cardiovascular effects associated with exposure to vinyl chloride or butadiene. The main reason for concern that aldehyde exposure could increase disease risk is that, by their very nature, some of these aldehydes are highly toxic. When delivered intravenously to animals, aldehydes in low doses (1-10 mg/kg) prolong Q-T interval and cause

arrhythmias and ventricular fibrillation. Depending on the dose, they could increase or decrease blood pressure (6). Aldehydes that stimulate stress responses or hormones increase blood pressure, however, by themselves they lower blood pressure. Indeed, one symptom of acute formaldehyde poisoning is a rapid fall in blood pressure, causing flushing, dizziness or even death.

Chronic exposure to aldehydes could have further adverse effects on cardiovascular health. For instance, it has been shown that animals fed aldehydes for several months develop hypertension, increase platelet calcium and show an increase in their heart weight, which is usually associated with poor cardiac performance (6). Aldehydes also inactivate important enzymes in the plasma which interferes with the function of HDL (the "good" cholesterol) and thus could elevate the risk of developing heart disease. Although such data strengthen the rationale for studying the cardiovascular effects of aldehydes, there is no firm evidence suggesting that humans exposed to aldehydes are at a greater risk for developing heart disease. However, our studies with animals exposed to aldehydes are beginning to provide some insights into how aldehydes could affect cholesterol levels, alter blood pressure and decrease the ability of the heart to protect itself from ischemia. Much work remains to be done, particularly in establishing the relevance of animal studies to human conditions.

Despite several unknowns in regard to the toxicologic nature of specific pollutants, it is clear that air pollution from current patterns of fossil fuel use is seriously affecting the health of millions of people worldwide. In addition, pollutants such as arsenic have caused arguably the worst toxicological disaster in human history in several developing countries (20-22). That air pollution could negatively impact human health is well established, however, the surprising feature of these new findings is that an increase in heart disease is the most significant and widespread outcome of pollutant exposure. This is borne out not only by the extensive burden of heart disease due to tobacco use, but also by significant increases in cardiovascular diseases in populations exposed to pollutants such as arsenic and ambient air particulates. Collectively, even by conservative estimates, pollution may be contributing to cardiovascular disease in several million individuals. These estimates underscore the urgent need to identify and control individual components of pollution that affect the heart and blood vessels. Studies underway aimed at determining how pollution affects heart disease will continue to enlighten and remind us of how delicate and entwined our health is with our environment.

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Not magic, but mass spectrometry to monitor interactions of environmental pollutants and the cardiovascular system

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Abstract.

As environmental cardiology continues to emerge as a subdiscipline, the tools of cardiovascular medicine and physiology are integrated with the tools of toxicology and epidemiology. For molecular level studies, the most powerful tool available is that of biomolecular mass spectrometry. This is a discussion of the historical developments and current applications of mass spectrometry that enable such use. Mass spectrometric techniques and analytical strategies are shown to be useful in molecular dosimetry studies, toxicokinetics and studies of mechanisms of action. Cardiovascular disease (CVD) is the number one cause of death in most developed countries, accounting for more deaths in 2001 in the United States than cancer, diabetes and accidents combined (Weinhold, 2004.) As with other diseases, a variety of causes can predispose or cause CVD, including genetic predisposition, as well as lifestyle factors which are more controllable, including diet, exercise and other good health care habits. In contrast to, for example, cancer, relatively little research has been accomplished examining potential roles of foreign compounds (xenobiotics) in causing or exacerbating CVD. The newly emerging field of environmental cardiology is beginning to address this area, with particular focus on the interactions of common xenobiotics encountered in developed urban settings, such as chemical pollutants derived from various manufacturing and shipping processes and products of combustion, including reactive xenobiotics, gasses, vapors and particulates. Some of these are known or suspected in the etiology of CVD.

Questions in Environmental Cardiology

Environmental cardiology raises a special set of toxicological questions concerning the interactions of xenobiotics and the cardiovascular system, including:

- What are the identities of the xenobiotics to which people are exposed?
- What is the nature of the exposure event? (Acute? Chronic? To what doses and by what routes are they exposed?)
- What is the fate of the xenobiotics when they enter the body? Where do they go? What are they changed to? How are they eliminated?
- What is the nature of the host-xenobiotic interaction? What new molecules are formed and what are their effects?

In order to study these phenomena at the molecular level, sophisticated tools are needed. Of course, cardiopulmonary medicine and related disciplines already provide tools of electrophysiology and sonography for study of physiological function that may be compromised by xenobiotic exposure. To study the suspect molecules from the environment, we have available the tools of analytic chemistry, especially those of chromatography and spectrometry.

The key molecules for study in environmental cardiology are those that result from biotransformation (metabolism) of xenobiotics and also newly formed molecules that result from chemical reaction of a xenobiotics or its derivative with proteins, nucleic acids (DNA, RNA), lipids (fats that make up much of biological membranes) and other biomolecules.

The development of tools needed for sophisticated analysis of the interactions between xenobiotics and complex biomolecules has undergone an explosion over the last generation. These tools include newly developed techniques in chromatography and electrophoresis, but the most important changes have been in application of the techniques of mass spectrometry to the questions of biomedical research.

Mass Spectrometry and its Development

Mass spectrometry (MS) has been used since the first experiments of J.J. Thompson in 1899 . In studying "positive rays", Thompson was able to maneuver such radiation with electromagnetic fields and to measure mass (For a brief history, visit the Scripps website http://masspec.scripps.edu/information/history/). Even to this day, the fundamental mode of operation of mass spectrometry remains the same as shown schematically in Figure 1:

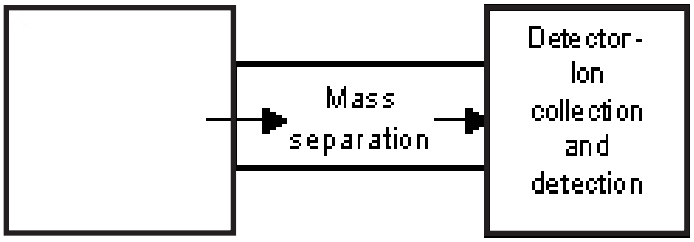

Figure 1. Schematic mass spectrometer. Although modern mass spectrometers are marvels of engineering, ion physics and chemoinformatics, the fundamentals remain the same.

Mass spectrometers are particularly useful because they are universal detectors. That is, the only requirement for an analyte is that it be made of matter. Mass spectrometers analyze matter in the gas phase in a charged state (ions). These ions (either positive or negative) are either introduced or produced in the ion source region. By applying a large voltage in this region, ions acquire kinetic energy and are accelerated through the mass analyzer. There are several types of mass analyzers which serve to measure, directly or indirectly, the momentum of these ions as they travel from source to detector. Momentum and kinetic energy are related directly to mass, and an attached data system is able to acquire data concerning the mass of these ions.

As we consider xenobiotics and biological molecules, it would seem that mass is not a unique identifying property. For example, nitrogen gas (N_2) and carbon monoxide (CO) each has a nominal molecular mass of 28 (14+14 or 12+16). What we have learned from chemistry, however, is that atoms do not have exactly integral mass values, and for this example, the true masses are for CO 27.995 and for N2, m/z 28.006. Many mass spectrometers can readily measure this difference and thus can distinguish the two gasses. This is equivalent resolution to that required to distinguish a 150 pound individual from another who weighs 150.06 pounds. In addition to this ability to measure masses very accurately, mass spectrometers have a very wide dynamic range, capable of mass measurement up to molecular masses of millions of Daltons.

To be useful for studies of part per billion pollutants diluted into 50-100 kg animals (adult humans), mass spectrometers must be very sensitive, able to measure small amounts of analyte. This is indeed the case, with some MS instruments capable of measuring 1000 or fewer molecules. This is very few indeed, as can be shown by a simple thought experiment. Take the U.S. national debt ($8.2 trillion and counting) and give that much money to every man, woman and child on the planet (6.58 billion and counting.) Try to imagine that amount of money, then realize that a teaspoonful of water has twice that many molecules of water!

Mass spectrometers have one more capability which gives them enormous power in structural analysis. Not only are MS instruments able to sensitively and accurately measure the mass of the molecules introduced into the ion source, but these instruments usually have a means for exciting analyte molecules to induce decomposition into fragments of these molecules. The masses of these fragments can be determined. This pattern is similar to a fingerprint, allowing one to deduce the structure of the analyte.

This ability to identify unknown compounds from crude mixtures of traces of analyte has lent a mystique to the use of mass spectrometry as a "magic black box" capable of detecting anything. Earlier television programs such as Quincy, M.E., and current programs such as C.S.I. and Law and Order are wildly popular, and often rely on the use of mass spectrometers. A small drop of fluid is introduced into the instrument (Figure 2) and instantly, a pattern of peaks appears on a computer screen, followed by dialog like, *"This spectrum clearly shows that the perp stopped at a truck stop in Tucson, had a bagel with apricot preserves and a skinny mocha latte immediately before raping and murdering Ms. Smith, who was wearing a lavender seersucker suit and chewing cinnamon gum at that time."*

"That's it, Johnson is our man. Bring him in."

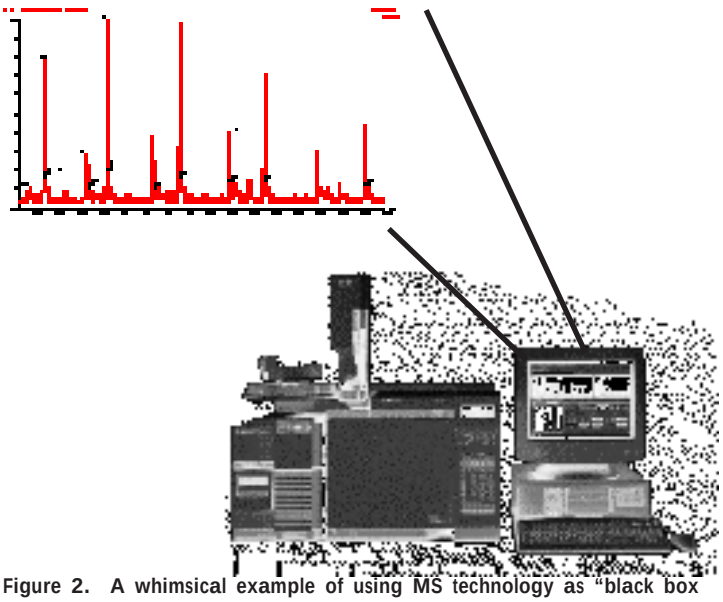

Figure 2. A whimsical example of using MS technology as "black box magic."

While this is very successful entertainment, it bears no resemblance to reality, and actually creates exaggerated expectations for mass spectrometry.

Modern Biomolecular Mass Spectrometry

All of the properties discussed above were available to scientists in the 1950s, yet biomolecular mass spectrometry (the application of MS to study of biomolecules) has only recently become commonplace. That is because such application awaited several developments in companion sciences.

Several advances in technology and prevailing scientific paradigm since the 1980s have enabled everyday use of mass spectrometry in biomedical sciences. These contributions are shown schematically in Figure 3.

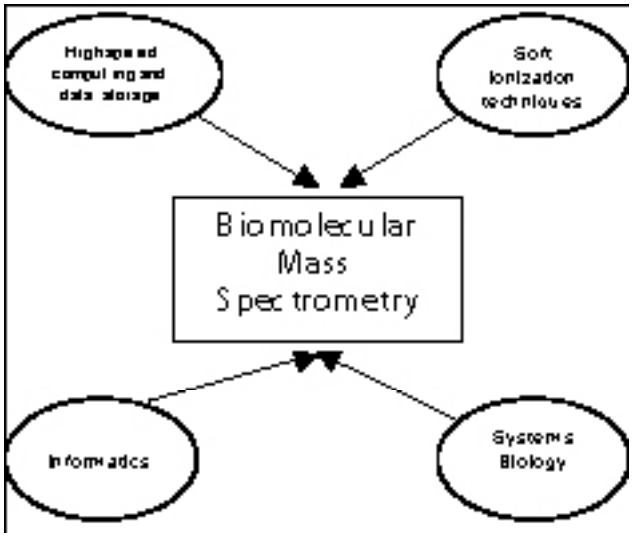

Figure 3. Scientific and technological changes since the 1980s have enabled everyday use of mass spectrometry in biomedical sciences.

Early mass spectrometers had paper strip output and mass assignments were made using a mix of pencils, rulers, graduate students and coffee. Although tedious, no other method could rival this. Modern mass spectrometers are operated using high speed computers with vast data storage capabilities, allowing sample collection in the GHz range (billions of scans per second.) Dramatic advances in data handling, and studies of very large data sets (see large scale biology below) have been the impetus for the rapid demand and growth in the fields of chemoinformatics and bioinformatics. These are disciplines which develop and use computational and statistical analysis of large data sets to infer chemical and biological meaning.

The need for new tools and approaches of informatics became very clear as the human genome project developed. The very large sets of data available were powerful tools in the hands of those able to use them. For this project, the goal was definition of the entire human genome – that is the structure of all of the genes and chromosomes. As this unfolded, other scientists argued that the relatively static genome serves as a blueprint, and while it is important, the complementary proteome (the structure of all of the proteins in a cell) is important in understanding regulatory biology. This is because the proteins are the molecules that provide structure in the cell and the proteins serve as catalysts to manage the chemical reactions within a cell. Finally, the discipline becoming known as metabolomics completes the picture, seeking to take a moment-to-moment snapshot of the smaller molecules (metabolites) in the cell. The attempt to study all of these phenomena simultaneously is called *systems biology*. Mass spectrometry is one of several technologies critical to this field.

The key technical advancement that has enabled biomolecular mass spectrometry is the development of so-called soft ionization techniques. Earlier MS instruments were not useful for proteins, nucleic acids, carbohydtaes and the like because of the harsh conditions used for vaporization, ionization or both. With the development of electrospray ionization (ESI) methods and laser desorption ionization (LDI) methods, this barrier was removed. These developments were sufficiently significant that John B. Fenn of Virginia Commonwealth University (ESI) and Koichi Tanaka of Shimadzu Corporation (LDI) received a portion of the 2002 Nobel Prize in Chemistry "for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules." Later work at the University of Frankfurt by Franz Hillenkamp and Michael Karas led to the development of a matrix. It is the development of ESI and MALDI that opened the door for biology to use mass spectrometry.

Aldehyde Pollutants and Analytical Strategy

Of the 300,000 or so sudden cardiac deaths each year, it is estimated that perhaps 20% are due to acute air pollution exposure. The cause and effect relationship, as well as the mechanisms of action are not clear.

The Environmental Cardiology team at the University of Louisville is examining a particular chemical class, the aldehydes, for their effects on molecular, cellular or systemic function. Aldehydes are a group of chemicals that can be found in high quantities in vehicle exhaust fumes and cigarette smoke. In this context, they are the products of incomplete combustion. In addition, these chemicals are also found in fried foods, fats and drinking water. The projects currently underway seek answers to four basic questions regarding the cardiotoxicity of aldehydes:

- How do cardiovascular tissues metabolize aldehydes and how does the cardiovascular system handle the challenge of foreign chemicals?
- How, and how quickly, do aldehydes accelerate the progression of heart disease?
- How do aldehydes interact with blood cells to cause cardiac and vascular inflammation?
- How do high levels of aldehydes raise the risk of heart attack and worsen heart failure?

The principal uses of mass spectrometry for molecular level analysis for these projects are in the areas of (1) molecular dosimetry, (2) toxicokinetics and metabolism and (3) mechanisms of toxicity.

Molecular Dosimetry

All of us are frequently exposed to a variety of aldehydes, from the environment (foods, water, air) and from normal metabolism, especially that of fats. These projects focus on the more highly reactive αβ unsaturated aldehydes, and particular attention will be paid to hexenal, 9-hydroxynonenal and acrolein. The need for a molecular dosimeter (a molecule whose presence and concentration reflect exposure) is several-fold. It is important to be able to mimic exposures that are realistic in comparison to real human exposures. It is important to know how to correlate exposure to risk of toxicity. It is important to have an analysis that allows us to monitor human exposure and to identify people at risk for developing cardiotoxicity. Finally, it is equally important to develop an analysis that can provide, with confidence, a means of reassuring the "worried well", those with potential exposure to aldehyde sources or at risk for cardiotoxicity who have not sustained a toxic exposure.

For molecular dosimetry and for inhalation toxicology in vivo we will develop a molecular dosimeter that will do one of the following:

- Quantitative measurement of acrolein in blood plasma using gas chromatography/MS analysis, or
- Quantitative measurement of small molecule that serves as "sink" for acrolein, but is readily excreted (for example, ascorbate or glutathione)
- Quantitative measurement of an extracellular protein that serves as "sink" for acrolein (this may be any abundant plasma protein)

These studies will be accomplished using GC/MS and LC/MS.

Toxicokinetics

For toxicokinetics, this project will identify and quantify metabolites of αβ unsaturated aldehydes, will study the rate of elimination of parent compound and metabolites, and will monitor especially those formed with ethanolamine, globin or albumin.

Mechanistic Studies

For mechanistic studies, understanding of chemistry of reactions of αβ unsaturated aldehydes and identification of targets will result in the following:

- Studies of mechanisms and kinetics of reactions of αβ unsaturated aldehydes and model peptides
- Performance of expression proteomics experiments to identify candidate proteins
- Use of cardiovascular function tests to suggest candidate protein targets
- Studies of mechanisms and kinetics of reactions of αβ unsaturated aldehydes and candidate proteins or nucleic acids

Analytical Strategies

Given the rich variety of mechanisms and potential number of target molecules, there are only a few different types of studies that will be performed. In anticipation of animals exposed via the inhalation route, preliminary experiments have been performed to demonstrate feasibility. These analyses are designed to study the fate of aldehyde air pollutants in the body: (1) GS/MS analysis for low molecular weight and volatile analytes, (2) expression proteomics, (3) model peptides and aldehyde reactivity, (4) MS protein analysis for identification of proteins and sites of adduct formation lducts.

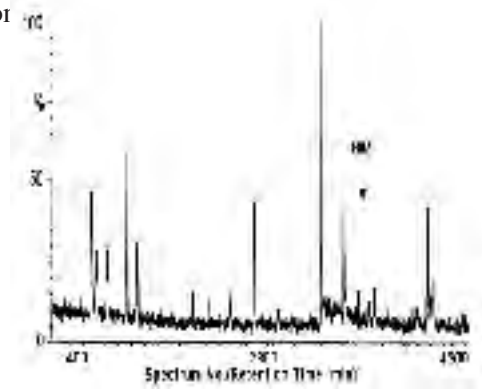

Figure 4. For this analysis of a single tissue extract, more than 4000 mass spectra were obtained. Each peak in this chromatogram corresponds to a single compound which can be identified by mass spectrometry (Srivastava et al., 1998).

GC/MS Analysis of low molecular weight species.

Methods for analysis of aldehydes using GC/MS have been published by this group and an example chromatogram is shown in Figure 4. In principle, each of these peaks can be identified using mass spectrometry.

GC/MS analyses are useful for a variety of applications in these projects. Examples include:

- Direct dosimetry – for some airbourne pollutants, measurement of the amount in the bloodstream will be the best measure of the delivered dose of the xenobiotics. Alternatively, endogenous metabolite adducts (lipids, carbohydrates, etc.) may be identified this way

- Exposure and risk – if this is indeed useful as a measure of delivered dose, then workers in the chemical industry, neighbors of such industry and others will have a simple test availale to demonstrate such exposure, which will guide treatment.

- The "worried well" – in the case of known or purported exposure to an area, there will almost always be a large group of the "worried well" those at risk who have not experienced significant exposure or risk. These can be reassured by negative findings of such tests.

MS Analysis of Protein-Adduct formation

Many xenobiotics are sufficiently reactive that they will form adducts with endogenous proteins. Such adducts are useful "biomarkers" of exposure. MS techniques are useful for discovery and monitoring of such biomarkers.

Model peptides and aldehyde reactivity - One approach is to study the chemistry of the reactivity of known aldehydes. Studies using acrolein and hydroxynonenal (HNE) have shown that the unsaturated aldehydes are indeed highly reactive toward peptide thiols and primary amines. For the following group of peptides and reactivity toward acrolein, the highlighted residue was the principal site of reactivity:

EGVYV**H**PV
P**K**PQQFFGLM-NH2
CDPGYIGSR
SVSEIQL**C**
RGP**C**RAFI

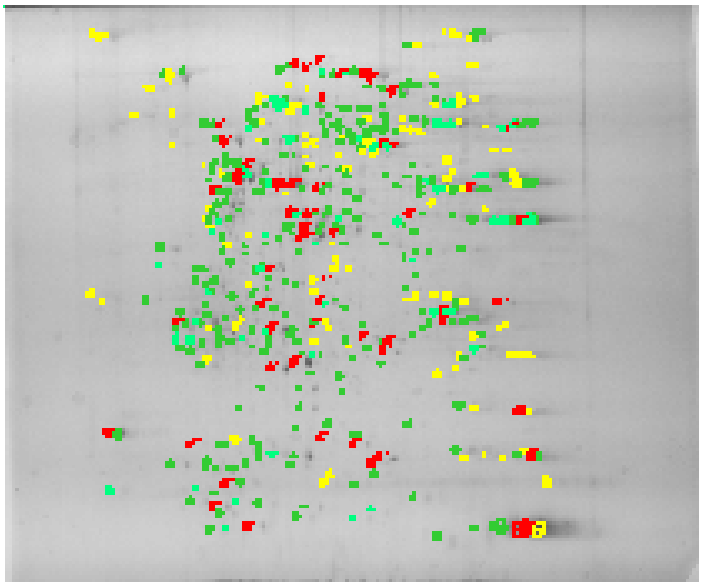

Figure 5. Proteins were extracted from tissues samples and separated using high resolution 2D PAGE analysis followed by MALDI-TOF mass spectrometric analysis of tryptic digests. Red dots designated protein "spots" that were identified using mass spectrometric patterns. Yellow spots have been selected for continuing study because the expression level is either higher or lower than control, but initial studies failed to identify them (Gozal et al., 2002)

Expression proteomics - One way to discover useful biomarkers formed as adducts of aldehydes is to attempt to study all available proteins from a tissue, before and after exposure to air pollutants. Data shown below in Figure 5 are from a report of the use of protein mass spectrometry to study oxygen sensitive pathways in regulatory biology. In this study, expression proteomics analysis using two dimensional electrophoresis and mass spectrometry was able to identify several altered proteins. After electrophoretic separation, each protein can be taken for MALDI peptide fingerprinting or further structural analysis.

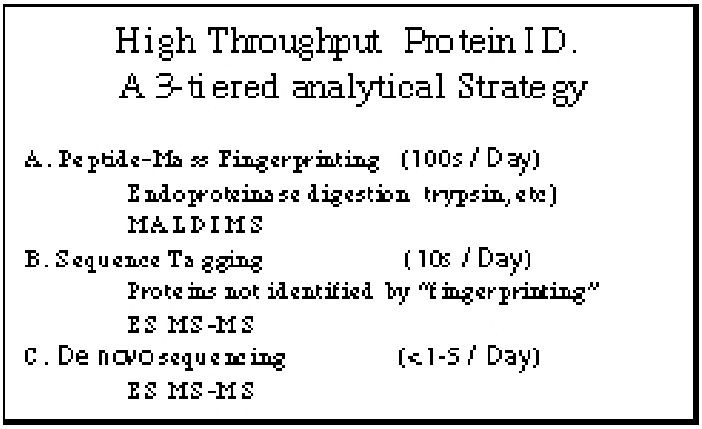

Figure 6

Peptide fingerprinting and partial sequencing

The use of MS techniques to identify proteins and study their structure, especially in cases of large scale biology design, is to obtain as much information as possible as efficiently as possible. Often a three tiered approach is used, as shown in Figure 6.

For peptide mass fingerprinting, a sample (usually excised from a gel or in solution as a column effluent) is treated with an endoproteinase, such as trypsin, which cleaves the protein into a few dozen smaller peptides. These are typically analyzed using MALDI and time-of-flight MS. The data obtained are the masses of the peptides. Since we know where the enzymes cleave (trypsin leaves an arginine or lysine on the carboxy terminus), we can go to a protein data base, and compare our data to all of the known proteins. A laboratory can perform hundreds of such analyses per day. Any adduct formed will have an altered mass and can be tentatively identified. If this approach is inadequate, tandem MS (two in-line MS systems) is used to determine the sequence of one or more peptides. Some proteins have as many as 500-1000 amino acid residues and typically no effort is made to determine all of these in a de novo sequencing study. If 7 consecutive residues in a peptide can be determined (this is often readily accomplished), this sequence can be used to interrogate a known genome (such as homo sapiens) asking he question "Where is this sequence found?" Even though this is a minor fraction of the protein's sequence, the random chance of finding 7 consecutive residues is less than 1 in a billion (20^7)! If this fails, de novo sequencing may be necessary. Combinations of these techniques yield identification of the protein, of the adduct and the site and amount of adduct formation.

As data accumulate concerning which proteins are modified by exposure to environmental compounds, molecular mechanisms can be deduced. For example, changes in inflammatory pathway proteins and/or lipid carrying proteins are likely to influence plaque formation and may increase the risk of myocardial infarction or stroke. Modifications of excitable cardiac tissue proteins or signal transduction proteins may lead to dysrhythmmias. Modifications of cytoskeletal proteins may underlie diminished cardiac output. This is the point for integration of physiological data and molecular level findings.

MS analysis of DNA-xenobiotic adducts

Reactive xenobiotics may form adducts with DNA as well. In some cases this will lead to diminished cell function, in others, to apoptosis or necrosis and in the worst cases, a mutagenic and ultimately a carcinogenic effect.

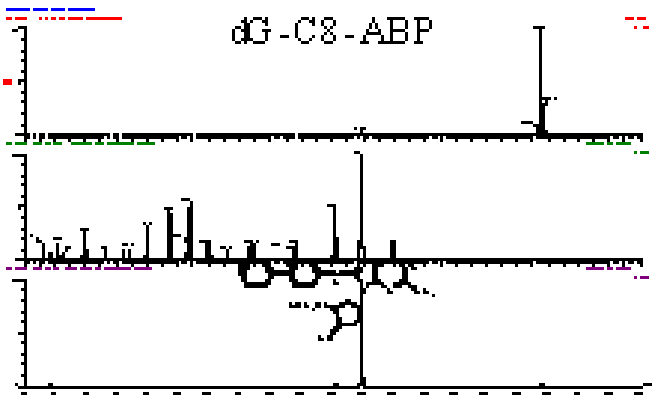

Figure 7

To examine this possibility, DNA samples were hydrolyzed to yield nucleosides, then taken for LCMS analysis to elucidate the structure of the aminobiphenyl adduct of deoxyguanosine as shown in Figure 7. (Neale, J.R., unpublished data.)Such analyses allow prediction or verification of mechanisms of toxicity.

While mass spectrometry is not a magical black box that can discover and prove everything (sorry CSI!) it is a very valuable tool for elucidation of toxicological mechanisms in environmental cardiology.

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