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Panel discussion review: session four — assessing biological plausibility of epidemiological findings in air pollution research

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In December 2006, the U.S. Environmental Protection Agency (EPA) sponsored a 2-day workshop on "Interpretation of Epidemiologic Studies of Multipollutant Exposure and Health Effects" in Chapel Hill, NC. The final session at this workshop was devoted to assessing the biological plausibility of epidemiological findings with regard to criteria air pollutants. The presentations and the panel contributions of this last session primarily focused on controlled exposure studies and led to wide-ranging discussions, some of which were provocative. The panel summary provides some guidance to future evaluations of the biological plausibility of the epidemiological reports on criteria pollutants and is intended to stimulate thinking, without drawing any definitive conclusions. This paper does not approach, nor was it intended to approach, the more formal analytical approach such as that used in EPA's development of its Integrated Science Assessment documents for the criteria pollutants.
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Background

Epidemiologic investigations have repeatedly demonstrated an association between human morbidity and mortality and exposures to ozone (O_3) and to particulate matter (PM) (EPA, 2004, 2006). Recently, population-level epidemiologic investigations have reported similar relationships between mortality and nitrogen dioxide (NO_2) and sulfur dioxide (SO_2).

One of the interesting dilemmas in evaluating air pollution health effects is the issue of assessing pollutant-specific effects to meet the Clean Air Act requirements, which center on individual pollutants. Although PM is increasingly recognized as a "mix", it too is still currently treated as a single entity that differs by size. Over the last 10 years, evidence regarding the association of air pollution and mortality and morbidity has been accumulating, often with specific statistical analyses of individual criteria pollutants occurring within the same study. Thus, "one study" is used to derive an understanding of O_3, PM, SO_2, NO_2, and/or carbon monoxide. This raises the question of how to use appropriately the same data sets for assessing several individual pollutants. A mortality event may be attributed to a specific pollutant in one study analysis and a different pollutant in another analysis, so that the same death may be "counted" multiple times. The answer to this dilemma may be to consider the data sets as a whole and evaluate the results in the context of biological plausibility.

The strongest evidence for health risks of an air pollutant results from convergence of information from epidemiological, human clinical, and animal toxicological studies. Each study type has its strengths and weaknesses (see Table 1), making an integrated interpretation of the results across disciplines the optimal approach. Understanding the health risks of exposure to O_3 has clearly demonstrated the value of such an integrated approach as the health effects and mechanisms are relatively well characterized. Data from controlled human and animal exposures to PM offer some suggestions that contribute to the understanding of the epidemiological reports implicating PM in mortality and morbidity. In contrast, the current database for NO_2 and SO_2 is less robust, especially for mortality.

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Table 1. Comparison of similarities and differences in the three main study approaches for criteria air pollutants.

	Laboratory animal studies alone	Human clinical studies alone	Epidemiological studies alone
Causal relationships	Yes	Yes	Unlikely
Specificity to individual pollutants	Yes	Yes	Not usually
Relevant concentrations	Can be close, but extrapolation needed	Close	Yes
Relevant exposure durations	Yes	Only for acute	Yes
Ambient mixtures	No, but emerging	No, but emerging	Yes
End points	Very specific, numerous	Very specific, several	Very broad
Subpopulations	Limited and extrapolation required	Limited	Very numerous
Mechanisms	Yes	Yes	No
Public health interpretation	Very difficult	Somewhat difficult	Yes

Discerning biological plausibility is quite difficult. Supporting information needs to be drawn from the full array of available literature (viz., epidemiology, human clinical, animal toxicology, and *in vitro*). Even then, a complete evaluation may not be possible based on the existing data. Fundamentally, population-level epidemiological studies involve a very large sample size (but heterogeneous population) with many confounding variables and rather general measures of mortality and/or morbidity, such as hospital admissions for broad classes of effects. Epidemiologic panel studies can provide additional insight into specific subpopulations by using sensitive clinical measurement methods. In contrast, controlled human exposure studies use rather homogenous populations with known exposures and discrete presumably sensitive end points, such as pulmonary function, cells in lung lavage fluid, and electrocardiogram (EKG). Consequently, a bridge needs to be established to achieve integration between controlled and observational studies. Insofar as databases converge, as with O_3, this linkage is somewhat easily ascertained, whereas with PM, the connections are still being elucidated. For SO_2 and NO_2, there are larger discrepancies between observational studies and the toxicological and clinical findings.

Seeking understanding of biological plausibility is crucial to risk assessment. Epidemiology can represent the real world, but can not establish causality. Controlled studies may readily show causality, but the study design is typically simple (e.g., one pollutant, one subpopulation, a narrow range of exposures, volunteers with good health or mild disease). So, effects due to ambient exposure scenarios that are not represented in a controlled study may go unrecognized. Animal studies can illustrate the full potential range of effects because a very large number of end points can be measured for various exposure paradigms, but ultimately the results must be extrapolated to humans, wherein substantial uncertainty exists. General outcomes of observational studies such as hospital admissions may be linked to laboratory findings of increased airway responsiveness, altered epithelial permeability, and inflammation. The optimal approach requires an understanding of the limitations of these approaches and integration across study types while recognizing and weighing similarities and differences (see Table 1).

The discussion to follow draws points from the various study approaches and then illustrates some of the factors and issues that need to be incorporated when seeking to gain insight into the biological plausibility of the adverse health effects of criteria pollutants. Although these considerations (pollutant mixtures, species and/or individual susceptibility, and health end points) are discussed separately for convenience of presentation, they have intricate interrelationships that must be treated as such for a more thorough and complete understanding.

Atmospheric mixtures

Two main categories of uncertainty surround the issue of air-pollutant-induced effects on the respiratory and cardiovascular systems: (1) extrapolating results of single pollutant, laboratory-based studies to complex ambient mixtures to determine the biological plausibility of epidemiological studies and (2) extrapolating results from complex ambient mixtures to individual pollutants to serve as the bases for the National Ambient Air Quality Standards.

An exceptionally strong database from controlled studies of a single pollutant can be very useful for the quantitative assessment of that single pollutant, but still leaves uncertainty for the ambient scenario because mixtures can affect health outcomes in numerous ways. Reactions between pollutants in a mixture may result in the generation or neutralization of a chemical species of real-world relevance that is not represented in the controlled exposure chamber. For instance, a sulfuric acid aerosol may be neutralized by ambient ammonia before inhalation or by endogenously derived ammonia after it is inhaled. Controlled studies of sulfuric acid seek to maintain the acid level by minimizing neutralization (e.g., acidic mouthwash before exposure). One (or several) chemicals in a mixture may also influence the dose of others in the mixture. For example, SO_2 may be adsorbed onto particles, thereby changing its regional respiratory tract

deposition. Madden et al. (2000) demonstrated that ozone-mediated oxidation of functional groups on diesel exhaust particle surfaces augments the oxidative stress and inflammation following instillation in rodents. At the biological level, one pollutant may make the target cell more sensitive to or enhance the harmful effects of another pollutant. Ozone may initiate oxidative stress, resulting in a cell that is less capable of defense against another co-occurring oxidative stressor, such as NO_2, or an infectious agent.

An implicit assumption in some statistical analyses of epidemiologic studies is that all ambient air pollutants are treated equally without regard to their potency. Therefore, health effects are often grouped with little regard to mechanisms and sites of deposition/absorption, biological processes, mechanisms of action, biological outcomes, and exposures; whereas specifics of individual pollutant concentration–response curves and dissimilar mechanisms of action should perhaps be considered. Concentration–response curves are very steep for O_3 relative to that for SO_2 or NO_2 and this difference should be reflected in the risk analysis. However, our understanding of biochemical mechanisms conferring individual susceptibility is still in its infancy. In addition, O_3 and NO_2 are oxidizing agents, whereas SO_2 is a reducing agent. Furthermore, NO_2 and O_3, are relatively water insoluble and SO_2 is soluble, resulting in different regional respiratory tract deposition patterns that would be expected to influence health effects. Moreover, with regard to NO_2 health concerns, factors such as endogenous production of NO_2 by peroxidases and reactive nitrogen chemistry need to be considered if one is to attribute significant health impacts from very low exogenous ambient exposures (e.g., 10 p.p.b.).

Animal toxicology studies have frequently relied upon an experimental approach in which models, typically rodents, are exposed to individual gaseous or particulate air pollutants. Significant exceptions exist — for example, animal models in a number of studies have been directly exposed in chambers to ambient air or a diesel combustion mixture and compared to control animals exposed to filtered air. Recent technological advancements have also permitted animal and human exposure studies of concentrated ambient particles (CAPs), with or without gaseous co-pollutants. Wellenius et al. (2004) provide one example of the cardiovascular effects of CAPs in a dog model of ischemia. During a 5-min coronary occlusion period, a consistent increase in the ST-segment elevation of the EKG waveform was observed in CAPs-exposed dogs compared to control dogs exposed to filtered air. Detailed analyses of the concentrated ambient PM provided information on physicochemical parameters including mass and number, sulfate, black carbon, elemental carbon, organic carbon, and trace element concentrations. Regression analysis of EKG waveform parameters with individual exposure parameter measurements, which differed on a daily basis, supported an association of ST-segment effects with airborne silicon, suggesting that earth crustal material played a role in the observed cardiac effects. Even so, the mechanism of this correlation remains elusive. This type of approach could provide useful information for identifying a responsible component(s) of complex mixtures in air pollution studies, although it must be remembered that CAPs are not equivalent to ambient PM exposures in terms of the particulate composition or proportion with co-existing gaseous pollutants.

Only a limited number of toxicology studies using ambient or simulated ambient mixtures have attempted to address the complex nature of exposure to ambient gases and particles and the diverse health effects associated with such exposures. In PM investigations, speciation data have been utilized to correlate changes in cardiovascular and allergy-related end points with specific components of ambient PM (Harkema et al., 2004; Morishita et al., 2004; Hwang et al., 2005; Lippmann et al., 2005). These studies have utilized repeated exposure protocols to take advantage of day-to-day changes in pollutant concentrations to assess specific toxicant components of the ambient milieu. In particular, the approach of Lippmann et al. (2005) has allowed quasi-time series studies of the adverse cardiovascular effects of PM and gaseous co-pollutants. These studies provide important information related to the biologic effects of relevant concentrations of gaseous and particulate air pollutants associated increases in morbidity and mortality.

Controlled animal studies of interactions between binary mixtures of ambient co-pollutants have not been particularly useful in terms of elucidating mechanisms of action or interactions. Examples drawn from studies of sulfuric acid and O_3 demonstrate that the current database is perplexing. Kleinman and Phalen (2006) observed concentration-dependent increases in lung lesions of rats exposed to O_3, but the co-exposure to sulfuric acid particles produced a concentration-dependent decrease in O_3-induced lung lesions (i.e., a quasi-protective effect). In addition, these same studies demonstrated that while O_3 caused a concentration-dependent decrease in macrophage function at a low concentration of sulfuric acid, the opposite occurred at a high concentration of sulfuric acid (i.e., a concentration-dependent increase in macrophage function). El-Fawal et al. (1995) also demonstrated complex concentration-dependent interactions between O_3 and sulfuric acid; some interactions appeared to be additive and enhanced airway effects, whereas others attenuated effects or were less than additive. Gardner et al. (1977) also showed that the temporal relationship of exposure to O_3 and sulfuric acid affects responses. Differences in protocols and results among such studies may represent different mechanisms and complicate the understanding of the adverse effects of criteria pollutant mixtures.

Gaseous co-pollutants in urban air have their own direct effects on the respiratory system and may affect particulate deposition as well. For example, an acute increase in airway obstruction is present during and immediately following short periods of exposure to ambient concentrations of O_3. This O_3-induced airway obstruction enhances deposition of aerosol (MMAD $\approx 2.5\,\mu$m) in the lower respiratory tract during tidal breathing at rest (Foster et al., 1993). Pulmonary inflammation and increased epithelial permeability following O_3 exposure may also lead to increased transepithelial transport of particles, which increases the risk of PM-related systemic effects and respiratory infections. Physiological changes (e.g., neuronal irritant reflexes, mucus secretion, non-homogeneous ventilation) are common acute-phase responses to respiratory irritants in the ambient aerosol mix and may also be capable of affecting particle deposition and subsequent adverse health effects. However, controlled exposure studies with a susceptible subject population (asthmatic individuals with inflammatory airway disease) have not demonstrated an enhanced acute-phase response for high ambient concentrations of submicron carbon particles in combination with ultrafine sulfuric acid aerosol (although some subjects did have an exacerbation of disease) (Anderson et al., 1992).

Population susceptibility

Populations can demonstrate substantial differences in response due to disparities in exposure (concentration and duration), delivered dose (as complicated by rates of deposition, clearance, metabolism, and biochemical reaction), and tissue sensitivity to that delivered dose. For example, individuals may have dissimilar doses delivered to target sites even when exposure conditions are identical. Assuming that delivered doses to target sites were equal, the responses will likely differ between individuals and across species. Similarities and differences between rodents and humans make quantitative extrapolation of exposure–response data from rodents on air pollutants difficult (Brown et al., 2005). Interindividual differences in humans complicate extrapolation from human clinical studies to epidemiological findings. The three main elements (exposure, dose, sensitivity) are discussed separately below, even though they are intricately linked in exposure–dose–response relationships.

Exposure

Exposure refers to the contact between an individual and the air pollutant(s) of interest over a particular duration. The most obvious difference between controlled and observational studies is the exposure chemical mixture, as already discussed. However, ambient mixtures have widely varying temporal and spatial components. Temporal differences range from minutes to years, whereas spatial differences range from city to city and within city and microenvironments (e.g., indoor, outdoor). All of this needs to be considered in evaluating biological plausibility. Animal research clearly shows major differences among acute, chronic, continuous, and intermittent exposures. Activity pattern data illustrate that a child has a different duration of outdoor exposure than an average adult or elderly person.

The relevance of exposure concentrations is always a critical factor in extrapolation of controlled studies, especially with animals, to observations in epidemiological studies. To elucidate toxicology mechanisms, studies with O_3, NO_2, and SO_2 frequently utilize concentrations that are generally much higher than those encountered in urban environments. In particular, ambient NO_2 and SO_2 concentrations are nearly two orders of magnitude less than the lowest concentrations that produce adverse effects in animal studies. The challenge of interpreting study results across this exposure concentration gap has several dimensions. First, the animal concentration may have been so high that it caused effects via mechanisms of action that would not exist in humans at ambient levels. Second, large epidemiological studies may have greater statistical power than small laboratory studies to detect effects at ambient pollutant levels due to the heterogeneity of the study population. Third, panel studies can target specific susceptible subpopulations and often employ personal monitors to get accurate estimates of exposure. Finally, the factors conferring susceptibility in humans may not have been elucidated and/or there may not be a suitable animal model to mimic human disease or risk other factors.

Dose and Dose Rate

Relevance of animal and human studies to biological plausibility cannot be based on exposure alone because exposure is not necessarily linearly related to delivered dose or dose rate. Comparisons between clinical and epidemiological studies are further complicated by non-linearity in the relationship between exposure concentration (C) and duration (T, time) and effect (i.e., similar $C \times T$ does not mean similar response). This is, in part, due to the nature of the reversible effects that are examined in controlled human exposure studies. For these reversible effects, such as decrements in forced expiratory volume in one second (FEV_1) or lavage fluid neutrophils, there are competing processes of injury and recovery (or repair) that determine the measured health effect. For instance, Jenkins et al. (1999) examined FEV_1 decrements and airway responsiveness to an allergen in a group of mild, atopic asthmatics. The subjects were exposed during rest for 6 h to filtered air, NO_2 (200 p.p.b.), O_3 (100 p.p.b.), and NO_2 (200 p.p.b.) plus O_3 (100 p.p.b.). The subjects were also exposed for 3 h to NO_2 (400 p.p.b.), O_3 (200 p.p.b.), and NO_2 (400 p.p.b.) plus O_3 (200 p.p.b.) to provide identical doses to the 6-h protocols

(i.e., equal $C \times T$). Immediately following the 3-h exposure, but not after the 6-h exposure, there were significant decrements in FEV_1 following O_3 and $NO_2 + O_3$ exposures. In addition, there were significant increases in airway responsiveness to allergen following all the 3-h exposures, but not following the 6-h exposures. Thus, even though the delivered $C \times T$ dose between 3- and 6-h exposures was identical, differences in the dose-rate and time for recovery led to different observed effects. Comparison of triangular exposures (ramping up O_3 concentration for first half of exposure and subsequently ramping down) and constant concentration exposures for O_3 have also shown that equivalent doses (equal $C \times T$) do not produce equivalent responses (Hazucha et al., 1992; Adams, 2006a, b). The importance of pattern of exposure has been amply demonstrated in mice exposed to NO_2 (EPA, 1993; Miller et al., 2000).

Although the relationships between exposure, dose, and effect vary across and within species, many of the factors affecting these differences are increasingly being recognized. Some key factors to be considered include morphology, physiology, and biochemistry of the respiratory tract, and physicochemical properties of the pollutant (Brown et al., 2005). For example, humans typically experience a range of breathing patterns during exposure to ambient pollutants, including increased minute ventilation during exertion as well as slower breathing frequencies during rest and sleep. However, animals are typically exposed during rest when they have relatively slower patterns of respiration. Rodents are obligate nose breathers, whereas most humans are oronasal breathers who breathe through the nose when at rest but who breathe increasingly through the mouth when activity levels increase and minute ventilation rises to meet metabolic demands. Lung morphology is species dependent. These dosimetric issues also become important in comparing human clinical and epidemiologic studies. Volunteers exercising heavily in a controlled environment would have a different deposition than if they were going about their daily life, which has far more variability.

Population Sensitivity and Potential Role of Mechanisms of Action

Knowledge of population sensitivity is growing. Factors affecting susceptibility include age (e.g., a developing lung may be more sensitive and exposure results in superimposition of damage/repair on top of growth; an aged lung may have less reserve capacity), pre-existing disease (e.g., coronary artery disease, asthma), species differences (e.g., rodents and humans may have different responses even if dose is equal), and interindividual differences (e.g., genetic or other unexplained variations in responsiveness).

Observations of Sensitivity Among similarly exposed individuals, matched for age and health status, there are differences in responses to inhaled ozone that are not explained by the relatively small differences in delivered doses (Ultman et al., 2004). Thus, the issue of interindividual variability and susceptibility becomes an important factor in evaluating the response curves of individual pollutants. These differences in response at some "level" of exposure appear to be log-normally distributed with variability increasing with the exposure concentration (McDonnell, 1996; McDonnell et al., 2007). An individual's FEV_1 response and/or inflammatory response on one occasion is related to their responses on a subsequent occasion; that is, those people who have a relatively extreme response with exposure, will likely experience a similar response when exposed again weeks or months later (Hazucha et al., 2003; Holz et al., 2005). Some of the interindividual variability in O_3 responses likely reflects innate susceptibility of the individuals due to genetic predisposition, diet, signaling pathways, immune responses, and other biological processes. The recognition of interindividual variability with some sensitive individuals having notable FEV_1 decrements supports the linkage back to the observation of adverse effects at generally lower ambient exposure conditions in epidemiological studies relative to those typically used in clinical studies.

The response within a single inbred strain of mice or rats is fairly homogeneous in comparison to human responses to inhaled particle and gases. Several recent studies have utilized across strain differences in rodent responses to examine the contribution of genetic host factors on pulmonary responses to certain air pollutants. Even if an outbred rodent population was used, by design, animal exposure studies frequently utilize small sample sizes that do not provide adequate power to statistically identify animals that differentially respond relative to their specific rodent population.

Asthmatics have somewhat augmented FEV_1 responses relative to healthy controls and these may increase with disease severity (Horstman et al., 1995). Human clinical studies with asthmatics suggested changes in pulmonary function and increased sensitivity to bronchoconstrictors after short-term exposures to near ambient (i.e., the upper limit of expected concentrations) NO_2 and SO_2 (EPA, 1993, 1994). However, the NO_2 clinical studies did not exhibit typical exposure–response relationships, as lower concentrations at times initiated effects, whereas higher concentrations did not. Furthermore, only some individuals were responsive within a given study. Generally, NO_2 clinical studies from several laboratories have been suggestive, but somewhat inconclusive, regarding observations of adverse health effects at relevant exposure concentrations. The results of SO_2 studies have been fairly conclusive and have shown that brief exposures (minutes) of asthmatics can cause pulmonary function effects and increased airway reactivity, interestingly these effects were not observed following longer duration (hours) exposure.

Ozone data clearly show the importance of interindividual variability, even among otherwise healthy populations. For the case of ozone-induced decrements in lung function, clinical and epidemiological evidence are fairly consistent. Clinical studies have successfully established that FEV_1 decrements during exposure to ozone with light activity levels are generally a function of the relationship between concentration, duration, and ventilation rate with FEV_1 decrements in healthy adults following exposure to 0.08 p.p.m. O_3 and perhaps as low as 0.06 p.p.m. O_3 (Ch 6, EPA, 2006).

Potential Mechanisms of Sensitivity In this section, the role of oxidative mechanisms is discussed to illustrate its potential role in species sensitivity and highlight some of the differences and similarities between controlled and observational studies. It is not intended to be a comprehensive discussion of mechanisms of action of criteria pollutants. In epidemiological studies, chronic conditions such as diabetes, coronary artery disease, asthma, and chronic obstructive lung disease have been shown to be associated with increased susceptibility to criteria air pollutants. In addition, animal studies recapitulate that "proinflammatory" states, such as diabetes, obesity, and antigen sensitization enhance susceptibility with regard to certain response markers. The issue of susceptibility of specific populations to air pollutants overlaps with hypothesized mechanisms. Many criteria air pollutant exposures induce an increased oxidative burden to the respiratory tract (O'Neill et al., 1995; Meng, 2003; Wang et al., 2006; Xia et al., 2006). Mechanisms vary but can include direct reaction-mediated generation of free radicals and secondary oxidants, upregulation of oxidases/peroxidases and nitric oxide, inflammation, and disruption of metal homeostasis. Therefore, susceptible subjects may be individuals with genetic or host deficiencies, who are less able to adequately maintain normal homeostatic controls that compensate for oxidant generation (e.g., glutathione reductase), inflammation (e.g., chronic granulomatous disease), and metal challenge (e.g., the elderly, diabetics, and those with coronary artery disease). Pre-existing obstructive lung disease may increase the deposition and/or alter clearance kinetics of PM; this is similar to a mechanism (i.e., increased dose rate and/or retention) by which risk can be altered.

Other groups, such as diabetics, have been identified as susceptible to adverse effects from inhaled PM in epidemiological studies, but have not been evaluated in controlled exposure studies. Despite this apparent limitation, toxicological, clinical, and panel studies provide some rationale for the increased susceptibility of diabetics. First, diabetics have decreased reactive oxygen species (ROS) defenses relative to healthy controls (Martin-Gallan et al., 2005). Inhalation of O_3, NO_2, and PM not only directly deliver oxidants to the lung surface but also result in additional oxidant production.

The lines between defense and mechanisms of toxicity can be complex. Pryor (1991) provides a thorough review on homeostasis and antioxidant defense to oxidant and PM exposures, covers chemical reactions, response data for both animal models and humans, and the topic of vitamin supplementation. Asthmatic children with a genetic deficiency of glutathione *S*-transferase are more responsive (greater FEF_{25-75} decrements) to O_3 exposure than asthmatic children without this deficiency (Romieu et al., 2004). In addition, antioxidant vitamins C and E attenuate O_3 responses in these children. In another study of asthmatic adults, this genetic deficiency was associated with increased allergic responses to secondhand smoke and diesel exhaust particles (Gilliland et al., 2006).

Epithelial lining fluid (ELF) antioxidants have been presumed to serve as a protective screen that scavenges extracellular free radicals, quenches oxidants, and limits lipid peroxidation. However, the relationships among supplementation, how these interventions may modify ELF antioxidants, and biological responses consequent to inhaled oxidant air pollutants are likely complex. For example, supplementation of athletic outdoor bicyclists with antioxidant vitamins confers partial protection against the acute effects of O_3 exposure and airway obstruction (Grievink et al., 1999). In a second study with controlled laboratory exposure to O_3 and a double-blind crossover design, antioxidant vitamin supplementation attenuated O_3-induced bronchial hyperresponsiveness in asthmatics (Trenga et al., 2001). In contrast to the above studies, vitamin C supplementation provided no protection against respiratory symptoms in a double-blind crossover design of healthy O_3-sensitive subjects (Mudway et al., 2006).

Animal studies have generally been unable to demonstrate protection by vitamin E (α-tocopherol) supplementation, except in animals initially depleted of vitamin E. However, species differences of antioxidant substances in ELF needs to be taken into consideration to gain perspective on susceptibility, in comparison and extrapolation to humans (Slade et al., 1993). Some rodent species/strains have higher concentrations of the major endogenous antioxidants than people (e.g., ascorbate), and, thereby, may be able to resist better the effects of ROS thought to be generated by or in response to pollutant exposures. Oxidative stress may be associated with other pulmonary end points such as inflammation and epithelial permeability and increases in either may alter susceptibility to PM-related toxic effects.

Another important issue, is the need to survey a number of lung responses. Human investigations with direct *in vivo* sampling of respiratory lining fluids have found protein levels of extracellular glutathione peroxidases to be predictive of inflammatory lung responses to O_3, but not of airway functional changes (Avissar et al., 2000). *In vitro* studies also

have bearing on this issue, and have recently shown that extracellular reactions of O_3 and NO_2 with antioxidants generate secondary reactive species that contribute to and/or cause pathophysiologic responses (Velsor et al., 2003; Connor et al., 2004; Ballinger et al., 2005). Thus, the precise contribution of ELF biochemistry to the myriad sequelae that occur in response to oxidant air pollutant exposures remains equivocal but is clearly complex and may, in part, contribute to individual and inter-species differential susceptibility.

Attributing oxidative stress to a single pollutant may or may not be valid since most measurements are obtained after inflammation has been initiated. Once inflammation (and/or upregulation of airway oxidases) occurs, there is little specificity to any oxidative stress measure. Elucidating connections between single air pollutants and oxidative stress in population-based studies is, at best, extremely challenging since numerous factors may influence the biological and physiological outcomes.

Health end points

Overall, there appears to be little information from the literature of controlled studies (in humans or animals) that suggests a rationale for the associations of human mortality with NO_2 and SO_2 in epidemiological studies. For example, laboratory animals exposed to more than 10 p.p.m. NO_2 or SO_2 exhibit many adverse effects, but no mortality, even if the exposure is prolonged. Much research exists on effects other than mortality. Epidemiological studies are typically limited to hospital records or other medical record databases and lead to generic end points such as hospital admissions or all-cause mortality. Linking the more sensitive and advanced human clinical and/or animal toxicological methods to such lumped indicators requires knowledge of mechanisms as well as rather general exposure–response findings. Panel studies provide opportunities for more direct analyses of human effects.

Human clinical studies have employed sensitive techniques to characterize a range of end points and their respective time courses. For example, Rusznak et al. (1996) demonstrated significantly increased allergen responsiveness in mild atopic asthmatics following exposure to NO_2 (400 p.p.b.) and SO_2 (200 p.p.b.) for 6 h relative to exposure to air. This airway hyperresponsiveness was immediate, but continued to increase until 24-h postexposure; the hyperreactivity remained elevated at 48-h postexposure. Delayed and/or prolonged effects of an exposure may not have been explored in many studies and so were missed. Even respiratory end points that appear independent may be due to inadequate measurements at different time points. For instance, inflammatory responses do not appear to be correlated with lung function responses in either asthmatic or healthy subjects exposed to O_3 (Balmes et al., 1996, 1997; Holz et al., 1999). However, the lack of correlation between inflammatory and spirometric responses may be due to differences in the time course of these different types of responses (Stenfors et al., 2002).

Table 2. End points that can be used in controlled human exposure studies of criteria air pollutants.

Pulmonary function
Spirometry
Diffusing capacity
Symptoms of breathing discomfort
Cough, pain on deep inspiration, throat irritation
Airway hyperresponsiveness
Specific challenges (e.g., allergen)
Non-specific challenges (e.g., cold air)
Cardiac responses
Blood pressure
Oxygen saturation
Electrocardiogram
Blood responses
Inflammation (e.g. C-reactive protein, Clara cell secretory protein)
Thrombosis (e.g. fibrinogen)
PO_2 saturation, hematocrit and viscosity
Peripheral blood monocyte activation
Serum antioxidants (e.g., α-tocopherol)
Bronchoalveolar lavage biomarkers
Epithelial permeability (i.e., total protein)
Inflammation (i.e., neutrophils)
Various cytokines
Urine
Isoprostanes
Exhaled breath and condensates
Volatile hydrocarbons and ROS
Acidity of condensates

Table 2 lists the range of human clinical end points. Many of these end points and more can be measured in animals. Animals offer additional opportunities to look for morphological changes, responses to infectious disease agents, chronic effects, and so on. Even so, the lowest levels at which effects have been observed in controlled exposure studies are exceedingly high compared to ambient concentrations, which are typically one or two orders of magnitude lower.

Discussion

For some criteria air pollutants and certain adverse health effects, strong concurrence exists across study approaches, especially for O_3. However, the difficulty of finding

concordance of epidemiological, human clinical, and animal toxicological results for some pollutants, such as SO_2 and NO_2, raises at least four possibilities to explain this discordance.

(1) NO_2 and SO_2 may be acting as a surrogate for a mixture, which is responsible for the adverse health effects observed in epidemiology studies. The majority of controlled studies of mixtures containing NO_2 and SO_2 have been binary mixtures (EPA, 1993, 1994). This database shows that binary studies do not elucidate the effects of these simple mixtures, much less realistic complex mixtures. For example, one set of binary mixtures can cause synergism, antagonism, and additivity; the response is likely dependent on ratios of concentration and timing of exposure. Such results demonstrate the importance of simulating realistic mixtures that occur in epidemiological studies. Hence, one should not expect a direct correlation between epidemiological and controlled studies, if the mixture is responsible for effects. When a single pollutant has observable effects with all approaches, as with O_3, one can expect a correlation that may be modified by the mixture.

(2) Different end points may have been studied. The current epidemiological results raising concern for inhaled ambient pollutants include mortality and cardiovascular morbidity, not just pulmonary morbidity. Pulmonary morbidity has some logical connections, the strength of which depends on the criteria pollutant. The issue of cardiovascular morbidity may have no *current* plausibility for NO_2 and SO_2 because controlled studies on such end points have not been performed using these gases. Some mixture studies are notable exceptions. The "Cincinnati dog" studies (EPA, 1980) measured cardiac end points after long-term exposures to mixtures containing NO_2 and SO_2. Recent research at NYU that employed CAPs and used susceptible animals have reported cardiovascular end points, but these studies do not have significant concentrations of NO_2 or SO_2.

(3) Epidemiological studies have populations with a far greater range of susceptibilities (by type and by severity) than represented in controlled studies. Since typically the epidemiological results show small shifts, it is conceivable that the shifts are driven by populations not included in controlled studies.

(4) The epidemiological findings may reflect some varying combination of the above. Thus, in total, it is suggested that results from population studies need to include a test of biological "reasonableness" in the context of the known exposure–response relationships, underlying mechanisms of action, documented outcomes in animal studies, and so on. In the absence of such, while the statistical analyses may point to one causative agent, erroneous conclusions may be reached. Biological systems and ambient exposures are complex. Epidemiological results are difficult to interpret because of the large diversity in populations and exposures. Thus, we should not expect a single, simple mechanism in clinical or toxicological studies to always explain or support epidemiologically observed health outcomes.

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