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Review

Conceptual model for assessing criteria air pollutants in a multipollutant context: A modified adverse outcome pathway approach

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ABSTRACT

Air pollution consists of a complex mixture of particulate and gaseous components. Individual criteria and other hazardous air pollutants have been linked to adverse respiratory and cardiovascular health outcomes. However, assessing risk of air pollutant mixtures is difficult since components are present in different combinations and concentrations in ambient air. Recent mechanistic studies have limited utility because of the inability to link measured changes to adverse outcomes that are relevant to risk assessment. New approaches are needed to address this challenge. The purpose of this manuscript is to describe a conceptual model, based on the adverse outcome pathway approach, which connects initiating events at the cellular and molecular level to population-wide impacts. This may facilitate hazard assessment of air pollution mixtures. In the case reports presented here, airway hyperresponsiveness and endothelial dysfunction are measurable endpoints that serve to integrate the effects of individual criteria air pollutants found in inhaled mixtures. This approach incorporates information from experimental and observational studies into a sequential series of higher order effects.

The proposed model has the potential to facilitate multipollutant risk assessment by providing a framework that can be used to converge the effects of air pollutants in light of common underlying mechanisms. This approach may provide a ready-to-use tool to facilitate evaluation of health effects resulting from exposure to air pollution mixtures.

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1. Introduction

Exposures to air pollutants, such as particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2), and sulfur dioxide (SO_2), have been linked to adverse respiratory and cardiovascular health outcomes (U.S. EPA, 2008a,b, 2009, 2013a,b). The U.S. Environmental Protection Agency has established National Ambient Air Quality Standards (NAAQS) to protect against health effects related to these pollutants, which are designated as criteria air pollutants or their indicator species under the Clean Air Act (Clean Air Act, 1990).

Criteria and other hazardous air pollutants are present in different combinations and concentrations across air sheds and within micro-environments. These complex mixtures of particulate and gaseous components are largely determined by local sources, long-range transport, atmospheric transformation, and local meteorological conditions. As a consequence, ambient air consists of an endless number of unique multipollutant mixtures, far exceeding the capacity of conventional epidemiologic or experimental approaches to characterize their health impact. The inability to adequately assess the impact of air pollution mixtures thus represents a significant gap in hazard assessment and will continue to foster uncertainty in risk characterization associated with exposure to ambient air. Strategies that minimize the need to assess a vast number of possible combinations are required to address this challenge.

New approaches are being developed for evaluating the health impacts of single pollutants in a multipollutant context and that of air pollution mixtures as a whole. The shift from a single to multipollutant approach has been encouraged by the National Research Council (NRC) and other scientists from academia, industry, and government (Johns et al., 2012). From an exposure science perspective, scientists have sought to achieve dimension reduction by identifying common physical or chemical properties shared by several components of the mixture and by generating models that address uncertainties related to the characterization of spatial and temporal variability of air pollutant mixture concentration profiles. In epidemiology, statistical techniques are being developed to evaluate mixtures of pollutants with similar biological properties. In toxicology, high throughput cellular and non-cellular assays are being used to increase the number of mixtures that can be studied in a narrow time-frame. Mechanistic evidence provided by these toxicological studies has implicated dozens of genes, biomarkers, proteins, and other factors. However, the utility of these findings is limited because of the inability to link changes to adverse outcomes that are relevant to risk assessment, such as measurable changes in organ responses, clinical consequences, and impacts to the population at-large.

Here we propose a conceptual model for assessing multiple criteria air pollutants based on the adverse outcome pathway (AOP) paradigm (Ankley et al., 2010). The AOP paradigm is a natural extension of other frameworks developed in the field of hazard and risk assessment for the purpose of characterizing the exposure to effects continuum. It synthesizes information relevant to the effects of a given chemical into a sequential series of steps which span multiple levels of biological organization. The AOP approach requires an understanding of mechanisms underlying the biological responses to pollutants of interest. Initiating events at the molecular level can include both specific receptor-ligand interactions and less specific events such as hydrophobic interactions between chemicals and cellular membranes. Results of experimental and epidemiologic studies can both be incorporated into this model. Thus, initiating events at the molecular level can be connected to adverse health effects at the individual or population level for which risk assessments are made.

Our approach extends the AOP model by requiring the identification of clinical endpoints that can be reliably measured in humans. It also requires that early events, even if caused by more than one mechanism, are upstream and predictive of clinical endpoints and health outcomes. Additionally, our approach groups pollutants by their ability to act through common mechanisms. Thus, we can use this model to integrate the effects of individual components of air pollutant mixtures at any converging intermediate endpoints along the pathway, irrespective of earlier events in the pathway.

In order to illustrate our approach, two case reports relevant to the effects of exposure to air pollution mixtures are presented below—one focusing on respiratory effects of irritant gases O_3, NO_2, and SO_2, and the other on cardiovascular effects of PM and O_3. Airway hyperresponsiveness (AHR) and endothelial dysfunction (ED), both measurable endpoints, are incorporated into AOPs that link early molecular and cellular changes to adverse population-based health outcomes.

2. Case report 1: irritant gases and respiratory outcomes

Respiratory health effects resulting from exposure to irritant gases SO_2, NO_2 and O_3 form the basis of NAAQS for sulfur oxides, nitrogen oxides, and O_3 and related photochemical oxidants, respectively (U.S. EPA, 2008a,b, 2013a,b). Experimental and epidemiologic studies demonstrate a wide range of effects from decrements in pulmonary function to increased visits to an emergency department or admission to a hospital. Some of these effects are related to asthma, which is a complex disease requiring a trigger such as an allergen or other stressor (O'Byrne et al., 2009). For an extensive review of evidence linking these criteria air pollutants to asthma, the readers are referred to recent Integrated Science Assessments (U.S. EPA, 2008a,b, 2013a,b). Key asthma-related findings and outcomes described in these documents are summarized below.

2.1. Definition of airway hyperresponsiveness

Enhanced airway responsiveness, here referred to as AHR, is a key feature of asthma, which is a chronic inflammatory disease of the airways (O'Byrne et al., 2009). Airway responsiveness reflects the sensitivity of airway smooth muscle to natural or pharmacological stimuli. It is measured in the clinic or laboratory by using a defined stimulus to challenge the airways in order to constrict the airway smooth muscle. This leads to airway narrowing and airflow limitation. Physiologic changes associated with variable airflow obstruction are usually measured in terms of forced expiratory flow in one second or specific airway resistance. Stimuli may be direct, i.e., act on specific receptors in the airway smooth muscle to cause constriction, or indirect, i.e., cause airway inflammatory cells to release mediators of bronchoconstriction. Methacholine and histamine are direct stimuli used in clinical and experimental

settings to assess AHR. Allergens, the most common inducer of AHR outside of the clinic, are indirect stimuli (Cockcroft and Davis, 2006). They mediate allergic responses characterized by an immediate immunoglobulin E (IgE)-dependent bronchoconstriction followed by a late asthmatic response. While the early asthmatic response typically begins within 30 min following allergen exposure, is responsive to inhaled β-adrenergic bronchodilators, and resolves within 3 h, the late asthmatic response typically develops during the 3–8 h period following exposure and is unresponsive to inhaled bronchodilators.

AHR is comprised of a "persistent" and a "variable" component, both of which contribute to physiologic measurements (Busse, 2010). Persistent AHR reflects structural changes due to airway "remodeling", such as sub-endothelial thickening, sub-basement membrane thickening, smooth muscle hypertrophy, matrix deposition, and altered vascular components, which occurs over time due to chronic inflammation. Variable AHR reflects acute inflammation present at the time of measurement. Variable AHR may be due to IgE-induced release of mediators from mast cells and basophils in allergic individuals or to other inflammatory mediators in non-allergic individuals. Some mediators (e.g., IL-13) and structural changes (e.g., smooth muscle hypertrophy) enhance the contractile response of smooth muscle to a given stimulus. Responses to air pollution may thus involve both persistent and variable components of AHR resulting, respectively, from chronic and acute inflammation.

2.2. Epidemiologic evidence

Epidemiologic studies have demonstrated positive associations between short-term exposures to ambient O_3 or SO_2 and respiratory symptoms, especially in children or asthmatic children (summarized in Section 6.2.4.5 (U.S. EPA, 2013b) and Section 3.1.4.8 (U.S. EPA, 2008b)). Respiratory symptoms assessed generally include cough, wheeze, and shortness of breath, consistent with asthma exacerbations. Evidence also indicated an association between short-term O_3 exposure and increased asthma medication use in children. A few studies found that atopic (i.e., having a genetic predilection to IgE-mediated immune responses) children and adults were at increased risk for SO_2-induced respiratory symptoms.

More severe respiratory effects associated with short-term O_3 exposure were reflected in increased respiratory-related emergency department visits and hospital admissions, including those for asthma (summarized in Section 6.2.9 (U.S. EPA, 2013b)). This was particularly the case in the warm season when ambient O_3 concentrations were higher. Evidence also indicated generally positive associations for SO_2 (summarized in Section 3.1.4.8 (U.S. EPA, 2008b)) or NO_2 (as summarized in Section 4.2.9 (U.S. EPA, 2013a)). Studies examining potential copollutant confounding provide evidence of independent effects of O_3 and SO_2 on respiratory-related outcomes. Effects of NO_2 on asthma-related respiratory outcomes that were independent of other traffic-related pollutants have also been demonstrated.

Studies of long-term exposures to O_3, SO_2, or NO_2, although fewer in number, have demonstrated associations with respiratory symptoms, bronchitis, or asthma (summarized in Section 7.2.8 (U.S. EPA, 2013b), Section 3.4.2.1 (U.S. EPA, 2008b), Section 5.2.17 (U.S. EPA, 2013a)). For SO_2, associations were found mainly in children and were less consistent. Long-term O_3 or NO_2 exposures have also been linked to new onset asthma in children. For O_3, this was associated with certain genetic variants. Interpretation of long-term studies is complicated by the fact that potential copollutant confounding was not often examined.

2.3. Evidence from controlled human exposure studies

Controlled human exposure studies demonstrated that inhalation of O_3 and NO_2 results in measurable amounts of lipid ozonation (Frampton et al., 1999) and lipid peroxidation products (Mohsenin, 1991), respectively, in the lung lining fluid. In addition, levels of S-sulfonates were increased in plasma following SO_2 exposure (Gunnison and Palmes, 1974). S-sulfonates are a reaction product of sulfite ion (which is formed by dissociation of sulfurous acid, the hydrated form of SO_2) with disulfide bonds.

Exposure to O_3 or NO_2 activated immune responses, such as neutrophil influx into the airways, in healthy individuals (Aris et al., 1993; Frampton et al., 2002). In addition, O_3 exposure enhanced the presence of cell surface molecules that are characteristic of innate immunity and antigen presentation on airway monocytes (Alexis et al., 2010). Repeated exposure to NO_2 had a pro-allergic influence, with increased expression of interleukins IL-5 and IL-13 in respiratory epithelium (Pathmanathan et al., 2003). These cytokines are characteristic of a T helper 2 lymphocyte (Th2) inflammatory response. IL-5 recruits and activates eosinophils and increases B lymphocyte production of IgE, while IL-13 promotes mucus production and AHR. Thus, NO_2 and O_3 exposure may contribute to the development of an allergic or asthmatic phenotype by enhancing antigen presentation and biasing the immune system towards a Th2-dominated response.

Immune responses were also enhanced in mild allergic asthmatics exposed to irritant gases. Numbers of airway eosinophils were increased in response to O_3 (Peden et al., 1997), NO_2 (Ezratty et al., 2014), or SO_2 (Gong et al., 2001). In addition, O_3 exposure resulted in greater expression of airway macrophage cell surface Toll-like receptor 4 (TLR4) and high- and low-affinity IgE receptors (Hernandez et al., 2010), and greater expression of bronchiolar epithelial IL-5 (Bosson et al., 2003) in allergic asthmatics compared with non-asthmatics. Levels of hyaluronan, an endogenous ligand of TLR4, were increased in lung lining fluid of asthmatics (Hernandez et al., 2010). NO_2 exposure followed by allergen challenge increased indicators of allergic inflammation, including numbers of airway neutrophils and levels of eosinophil cationic protein (ECP) in lung lining fluid of asthmatics (Barck et al., 2002). In subjects with allergic rhinitis, NO_2 exposure enhanced ECP levels in nasal lavage fluid following challenge with an allergen (Wang et al., 1995). Thus, exposure to irritant gases may exacerbate allergic responses in previously-sensitized individuals.

Exposure to irritant gases resulted in physiologic changes in airway smooth muscle. SO_2 inhalation increased airway resistance, especially in adult asthmatics (Johns and Linn, 2011). Modest increases in airway resistance occurred in healthy humans exposed to O_3 (Hazucha et al., 1989) and in chronic bronchitics exposed to NO_2 (von Nieding and Wagner, 1979). Pharmacologic studies provided insight into the mechanisms underlying these responses. Pretreatment with the anticholinergic agent atropine blocked the SO_2 response in healthy humans, indicating that acetylcholine release by airway nerves, specifically vagal cholinergic parasympathetic pathways, mediated the increased airway resistance (Snashall and Baldwin, 1982). However in asthmatics, the response to SO_2 was only partially blocked by anticholinergic agents (Myers et al., 1986). Partial inhibition of SO_2-induced increases in airway resistance by cromolyn sodium, a mast cell stabilizing agent, suggested that inflammatory mediators released by mast cells (e.g., histamine) also contributed to the response in asthmatics. Atropine inhibited O_3-induced increases in airway resistance in healthy humans (Beckett et al., 1985), but failed to inhibit NO_2-induced increases in airway resistance in chronic bronchitics (von Nieding and Wagner, 1979). However, a histamine-suppressing agent blocked the NO_2 response. Thus, experimental evidence indicates that both neural reflex responses and inflammatory

mediators mediate physiologic changes in airway smooth muscle following exposure to irritant gases.

Furthermore, inherent reactivity of airway smooth muscle was enhanced by exposure to O_3 or NO_2 in healthy and asthmatic subjects. The increased bronchial reactivity (i.e., AHR) to a direct challenge (e.g., methacholine) was seen in exercising asthmatic and non-asthmatic individuals following O_3 exposure. The AHR response to O_3 persisted 18–20 h following exposure (Foster et al., 2000), which is consistent with epidemiologic evidence of increases in hospital admissions for respiratory diseases 1 day after peak levels of ambient O_3. Increased bronchial reactivity to direct challenge agents was also seen following NO_2 exposure in exercising and non-exercising subjects, with asthmatics exhibiting greater sensitivity than non-asthmatics (Folinsbee, 1992). Pharmacologic studies demonstrated that atropine blocked the increase in O_3-induced bronchial reactivity (Holtzman et al., 1979). Supplementation with the antioxidant ascorbate prevented AHR in asthmatic subjects exposed to NO_2 (Mohsenin, 1987). Thus, enhanced activity of airway nerves and redox status may influence airway responsiveness following exposure to O_3 and NO_2, respectively.

AHR to allergens occurred in mild allergic asthmatics following exposure to O_3 (Jorres et al., 1996), NO_2 (Tunnicliffe et al., 1994), or concurrently to SO_2 and NO_2 (Rusznak et al., 1996). In the case of NO_2 exposure, both exacerbated early and late phase asthmatic responses were observed. In the case of concurrent SO_2 and NO_2 exposure, AHR persisted for 48 h. Thus, exposure to irritant gases may exacerbate allergen-provoked asthma in previously-sensitized individuals.

2.4. Evidence from toxicological studies

Studies in experimental animals support and extend the findings of controlled human exposure studies that showed that exposure to irritant gases resulted in (1) the presence of reaction products in lung lining fluid, (2) activation of immune responses, (3) physiologic changes in airway smooth muscle and (4) inherent changes in smooth muscle reactivity. These results are briefly described.

Formation of hyaluronan fragments, nitrated surfactant protein D, sulfite, and S-sulfonates in lung lining fluid occurred in experimental animals exposed to O_3, NO_2, and SO_2 (Garantziotis et al., 2010; Matalon et al., 2009; Gunnison et al., 1981). In addition, activation of immune responses, including allergic sensitization in naïve animals and exacerbated allergic responses in allergen-sensitized animals, occurred following inhalation of O_3 (Hollingsworth et al., 2010; Larsen et al., 2010), NO_2 (Bevelander et al., 2007; Gilmour et al., 1996), or SO_2 (Riedel et al., 1988; Song et al., 2012). Activation of the TLR4 pathway, dendritic cell maturation, and polarization to Th2 and Th17 phenotypes were implicated as mechanisms underlying the adjuvant effects of O_3 and NO_2. Furthermore, bronchoconstriction was demonstrated in experimental animals exposed to SO_2 (Nadel et al., 1965). This response was due to activation of bronchial C-fibers and cholinergic parasympathetic pathways involving the vagus nerve. Moreover, exposure to O_3 or NO_2 resulted in increased airway smooth muscle reactivity (i.e., AHR) (Hollingsworth et al., 2004; Kobayashi and Shinozaki, 1990). Experimental evidence showed that O_3-induced AHR resulted from hyperreactivity of the vagal nerves (Freed et al., 1996). Sensitization of C-fibers by inflammatory mediators (Lee and Widdicombe, 2001) or the release of major basic protein by eosinophils, which promotes acetylcholine release from parasympathetic nerves, (Yost et al., 2005) may also contribute to O_3 induced-vagal hyperreactivity. Other studies suggested that stimulation of local axon reflexes in which C-fibers release tachykinins (Joad et al., 1996) or disruption of the epithelial–mesenchymal unit during lung development may play a role in O_3-induced AHR (Plopper et al., 2007). Experimental evidence suggested that mast cell degranulation, allergic inflammation, and airway remodeling may mediate AHR resulting from NO_2 or SO_2 exposure (Thomas et al., 1967; Kobayashi and Miura 1995; Song et al., 2012).

2.5. Mechanisms underlying airway hyperresponsiveness

Emerging evidence in humans and animal models of allergic airway disease indicates that endogenous reactive oxygen and nitrogen species (ROS, RNS) play a role in AHR. ROS and RNS are produced by respiratory tract cells including epithelial, dendritic, T lymphocytes, macrophages, neutrophils and eosinophils, especially during inflammation (Ckless et al., 2011). Oxidative products of activated neutrophils and eosinophils have been demonstrated in lung lining fluid and tissue of asthmatics (van der Vliet, 2011). These markers correlated with the degree of inflammation and/or severity of clinical symptoms. In addition, individuals with asthma had higher levels of reactive nitrogen metabolites and greater numbers of ROS-producing cells in lung lining fluid compared with healthy individuals (Anderson et al., 2011). Upregulation of inducible nitric oxide synthase (iNOS) was also observed in airway tissue of asthmatics. These changes suggest that enhanced production of superoxide anion and NO, which react to form peroxynitrite (a key mediator of nitrative stress), contribute to the asthma phenotype.

Furthermore, studies provide evidence of a link between allergen challenge and ROS/RNS. The oxidative stress marker, 8-isoPGF2α, was elevated in asthmatics following allergen challenge and in asthma exacerbations (Voynow and Kummarapurugu, 2011). Allergen challenge increased protein tyrosine nitration, a marker of nitrative stress, in the airways of asthmatics (Comhair and Erzurum, 2010). Similarly, increased protein tyrosine nitration was found in lungs of animals with allergic airway disease. Moreover, allergen challenge resulted in a rapid increase in 8-isoPGF2α, a decline in the antioxidant reduced glutathione, and an increase in oxidized glutathione in lungs of allergic animals (Kloek et al., 2010). These changes correlated with onset of airway obstruction. Other experimental evidence suggested a relationship between ROS/RNS and destabilization of mast cells, leading to release of histamine and other mediators which provoke airway obstruction or activate T lymphocyte subsets (e.g., $CD4^+$).

Experimental studies also show that modulating redox status influenced airway responsiveness. In an animal model of allergic airway disease, depletion of glutathione enhanced airway responsiveness to histamine, while augmentation of glutathione reduced it (Kloek et al., 2010). Similarly, glutathione augmentation reduced airway responsiveness to an allergen (Koike et al., 2007). Furthermore, reducing peroxynitrite formation in inflamed lungs decrease AHR (Mabalirajan et al., 2010).

Evidence indicates that redox status also influences allergic responses. In allergen-sensitized animals, augmentation of lung glutathione or reduction of peroxynitrite resulted in decreased eosinophil infiltration into the lung, decreased levels of Th2 cytokines and chemokines, and increased levels of IL-12, a Th-1 pathway marker, in lung lining fluid in response to allergen challenge (Koike et al., 2007). These findings suggest that a shift in Th1/Th2 balance away from Th2 and inhibition of eosinophil recruitment occurred as a result of decreased oxidative/nitrative stress. Other steps involved in allergic sensitization, such as maturation of antigen presenting capacity of dendritic cells and dendritic cell stimulation of $CD4^+$ lymphocytes, may also be sensitive to redox status (Ckless et al., 2011).

Endogenous ROS/RNS may further impact AHR by activating redox-sensitive transcription factors involved in inflammation

(Comhair and Erzurum, 2010). Redox reactions may promote apoptosis of airway epithelial cells and lead to airway remodeling (Comhair and Erzurum, 2010). For example, reducing peroxynitrite-mediated nitrative stress reversed airway remodeling in allergic airway disease (Mabalirajan et al., 2010). Airway remodeling and persistent inflammation may contribute to AHR by enhancing the contractile response of smooth muscle. This can occur due to structural changes in the airway walls or through release of mediators such as IL-13 (Cockcroft and Davis, 2006). Thus, ROS/RNS may contribute to acute inflammation, which underlies variable AHR, and to chronic inflammation and airway remodeling, which underlie persistent AHR.

2.6. AOP: air pollution mixtures and respiratory outcomes

Inhaled irritant gases may exacerbate asthma and allergic airway disease by adding to the lung burden of ROS and RNS. Further, they may contribute to the development of an allergic and/or asthma phenotype by influencing redox-sensitive pathways involved in allergic sensitization and airway remodeling. These steps are outlined in the following proposed AOP.

As depicted in Fig. 1, inhalation of O_3, NO_2, and/or SO_2 leads to redox reactions in lung lining fluid and/or tissue. This includes formation of secondary oxidation, nitration, and sulfitolysis products such as lipid peroxidation products, arachidonic acid metabolites, ozonized lipids, hyaluronan fragments, nitrated proteins, sulfites, and S-sulfonates. Transient depletion of antioxidants and altered redox state may also occur. These macromolecular interactions comprise the molecular initiating events in the AOP. These processes may be amplified by ROS/RNS generated by recruited and activated inflammatory cells, resulting in a positive feedback loop.

At the cellular response level, multiple pathways may be triggered including those that facilitate release of acetylcholine or histamine. For example, reaction of secondary oxidation products with chemosensitive receptors on nerve fibers may activate vagal neural reflexes leading to acetylcholine release. Alternatively, secondary products, such as eicosanoids, may sensitize chemosensitive receptors, resulting in neural reflex responses triggered by lower concentrations of agonists. Redox reactions may activate eosinophils resulting in degranulation and release of major basic protein which enhances acetylcholine release by post-ganglionic vagal fibers. Furthermore, lipid peroxidation of mast cell membranes may promote degranulation and release of histamine or other mediators of smooth muscle contraction.

Products of redox reactions may activate redox-sensitive transcription factors involved in inflammation. Redox reactions may generate hyaluronan fragments or other products which stimulate TLR signaling in airway epithelial cells and inflammatory cells. Steps involved in allergic sensitization may be stimulated by the TLR pathway or an altered redox state. Moreover, formation of secondary products may promote injury or apoptosis of airway epithelium. In the developing lung, subtle injury may disrupt the epithelial–mesenchymal unit, which is critical for development of airway structure and function.

Cellular changes may lead to organ responses. This includes an inherent increase in airway smooth muscle contractility or sensitivity to agonists such as acetylcholine and histamine, which are characteristic of AHR. Upregulated expression of cytokines and chemokines may mediate acute airway inflammation. Inflammatory mediators may enhance smooth muscle contractility by sensitizing nerve fibers and facilitating acetylcholine release or by altering the contractile mechanisms of airway smooth muscle. Mediators released by mast cells may promote allergic inflammation. Activation of TLR signaling pathways may enhance immune responses to triggers such as allergens and endotoxin. Recurrent activation of the TLR pathway may lead to allergic sensitization and/or allergic inflammation. Epithelial injury may increase airway permeability which allows greater access of allergens to immune cells or greater access of mediators to sensory receptors on nerve fibers underlying the epithelial layer. Recurrent epithelial injury may promote chronic airway inflammation and remodeling, thereby increasing airway smooth muscle contractility. In the developing lung, prolonged disruption of the epithelial–mesenchymal unit may compromise airway growth and development. All of these processes may contribute to AHR.

At the individual response level, reversible airflow obstruction and respiratory symptoms, which are characteristic of asthma exacerbation, may occur in the presence of a trigger. These responses may be more exaggerated and occur with less provocation than in the absence of AHR. Furthermore, the

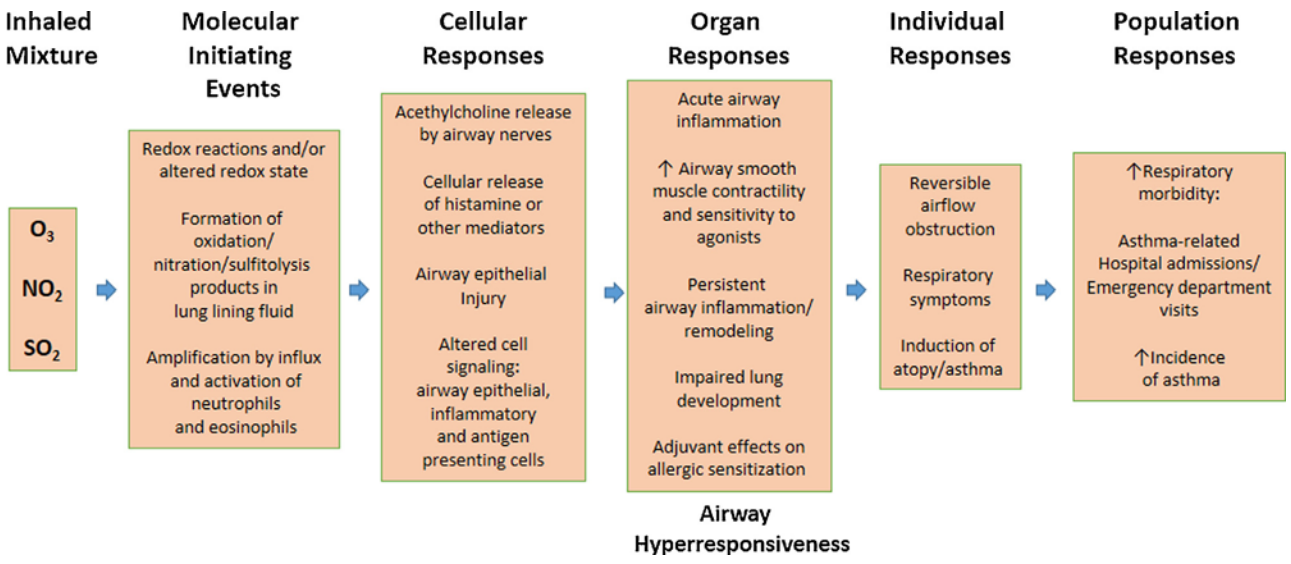

Fig. 1. AOP: air pollution mixtures and respiratory outcomes.
This AOP depicts a sequential series of higher order effects linking exposure to NO_2, O_3, and SO_2 to an adverse outcome with relevance to risk assessment, i.e., airway hyperresponsiveness.

acquisition of an allergic phenotype may lead to de novo induction of atopy and/or allergic asthma.

Increased asthma exacerbations, an important clinical outcome, may be reflected at the population level by increased numbers of emergency department visits and hospital admissions. These responses may be immediate or delayed due to the time required for immune system activation. Responses converging on induction of AHR or atopy/allergic asthma may lead to increased incidence of cases of new onset asthma in the population.

3. Case report 2: particulate matter, ozone, and cardiovascular outcomes

Epidemiologic studies have demonstrated associations between air pollution exposures and cardiovascular morbidity and mortality. Of all the pollutants linked to cardiovascular health effects, the evidence for fine PM ($PM_{2.5}$) is strongest, suggesting that PM is both a trigger of acute events and an initiator of biological responses that cause disease or promote its progression (Brook et al., 2010). Subgroups including the elderly and those with pre-existing cardiovascular disease appear to be the most susceptible, with PM exposure exacerbating the cardiac and vascular pathophysiology attendant to hypertension, ischemia, heart failure, diabetes, coronary artery disease and other dysfunctions. According to the Integrated Scientific Assessment for PM (U.S. EPA, 2009), the most recent statement on PM by the American Heart Association (Brook et al., 2010), and the recent position paper by the European Society of Cardiology (Newby et al., 2015), the preponderance of evidence points to a causal relationship between $PM_{2.5}$ exposure and increased cardiovascular morbidity and mortality. Both short- and long-term exposure to $PM_{2.5}$ are associated with increased cardiovascular risk, with apparently greater risk associated with long-term exposure. Among the irritant gases, the cardiovascular effects of O_3 exposure have been the best studied. Although emerging data implicates O_3 as a cardiovascular threat, the information available pales in comparison to that of PM.

A consideration of mechanisms underlying the cardiovascular effects of PM and O_3 suggests some overlap. Several mechanisms of PM-induced cardiac dysfunction in humans have been postulated including autonomic modulation, direct effects of PM constituents on cardiomyocyte ion channels, and vascular dysfunction related to systemic inflammation (Brook et al., 2010). Less is known, however, about mechanisms mediating O_3-induced cardiovascular responses (U.S. EPA, 2013b). Preliminary evidence implicates altered autonomic tone, systemic inflammation, vascular oxidative stress, and endothelial/vascular dysfunction (Srebot et al., 2009).

The link between respiratory effects due to PM or O_3 exposure and systemic effects is not well understood. The penetration of inhaled PM into the lung can cause pulmonary injury resulting from interaction of particles with epithelial cells or from phagocytosis of particles and a resultant oxidant burst (Brook et al., 2010). O_3 exposure results in lipid peroxidation of cellular membranes and the formation of other secondary oxidation products in lung lining fluid (U.S. EPA, 2013b). Injury from these pollutants leads to release of chemoattractants and inflammatory mediators that drive the influx of inflammatory cells such as neutrophils into the lung, perpetuating the production and release of inflammatory mediators. Some evidence suggests that local mediators of inflammation (e.g., IL-6) spill over into the general circulation and trigger systemic inflammation, including liver production of acute phase proteins such as C-reactive protein and fibrinogen, and vascular oxidative stress, including up-regulation of the superoxide anion-generating enzyme NADPH oxidase (Brook et al., 2010).

3.1. Definition of endothelial dysfunction

ED is generally defined as an impaired blood vessel response to specific vasodilators, including bradykinin and acetylcholine (Endemann and Schiffrin, 2004). ED can take place both in conduit arteries, large vascular segments whose principal function is to direct oxygen and nutrient-rich blood flow to target organs (e.g., coronary arteries, brachial artery), and in microvascular resistance vessels (i.e., arterioles), which are the principal determinants of peripheral vascular resistance. Under normal physiological conditions, the endothelium, which is a single layer of cells on the luminal surface of blood vessels, mediates vascular dilation to maintain normal blood pressure and ensure adequate organ perfusion, prevents platelet activation and clot formation, and inhibits leukocyte adhesion (Madden, 2012). Injury to the endothelium has several untoward effects including promotion of vasoconstriction, thrombosis, and inflammation (Rajagopalan et al., 2005). These effects can lead to atherosclerotic plaque progression and coronary vasospasm, and, ultimately, myocardial ischemia, as well as vascular remodeling and hypertension (Madden, 2012). ED may occur when factors that promote vasoconstriction, thrombosis, and smooth muscle cell proliferation overwhelm factors that maintain homeostasis and normal endothelial function. The most critical among factors involved in preserving normal endothelial function is NO, which in addition to being a potent vasodilator, has anti-thrombotic, anti-inflammatory, and anti-proliferative properties particularly in arteries with smooth muscle (e.g., coronary arteries). Other key endothelial-derived factors that promote vasodilation include prostacyclins and endothelium-derived hyperpolarization factors, although they tend to play a more important role in smaller vessels (i.e., microcirculation). The hallmark contributing factor to endothelial dysfunction is loss of NO bioavailability. This can occur as a result of decreased NO synthesis or increased loss of NO due to scavenging by reaction with superoxide anion (Rajagopalan et al., 2005).

Many risk factors for cardiovascular disease are associated with ED, which is itself an independent predictor of future cardiovascular events (Flammer et al., 2012). ED is associated with and often precedes a spectrum of cardiovascular diseases including hypertension, coronary artery disease, congestive heart failure, renal failure, types 1 and 2 diabetes, and atherosclerosis (Endemann and Schiffrin, 2004; Flammer et al., 2012). In the latter case, ED contributes to atheroma formation in coronary and peripheral arteries, and to plaque progression.

Many methodological approaches have been developed to measure the physiological function of the endothelium in humans. These include invasive techniques such as venous occlusion plethysmography and noninvasive techniques such as flow-mediated dilatation (FMD) and peripheral arterial tonometry that use the brachial artery in the arm as a surrogate for coronary circulation (Flammer et al., 2012). The fundamental principle with all these approaches is the assessment of arterial responses to either the release of temporary physical occlusion (reactive hyperemia) or pharmacological stimuli including vasodilators (e.g., acetylcholine). These methods have proven effective in demonstrating reduced function in disease states. Impaired vascular function independent of the endothelium, such as dysfunction of smooth muscle, can also be measured (e.g., nitroglycerine).

3.2. Epidemiologic evidence

Multiple studies link ED with cardiovascular risk of atherosclerosis, hypertension and diabetes mellitus. Many, but not all, studies of short- and long-term exposures to PM have found associations with ED. Results of panel studies involving short-term exposures

are mixed. Exposures to particles (black carbon and sulfate) from coal-burning power plants and traffic were associated with impairment of endothelium-dependent and -independent vasodilation in diabetics (O'Neill et al., 2005). In addition, Dales et al. (2007) found that reduction in FMD was associated with a 30 μg/m^3 increase in $PM_{2.5}$ in healthy individuals exposed while waiting at bus stops. However, Bräuner et al. (2008) found that exposure to particle-rich urban air over a 24-hour period had no significant impact on microvascular function in healthy subjects. Krishnan et al. (2012), using data from the Multi-Ethnic Study of Atherosclerosis (MESA), a prospective study that examined the prevalence and progression of subclinical atherosclerosis in middle-aged to elderly individuals, examined the relationship between short- or long-term PM exposure and endothelial function, which was assessed by FMD and baseline arterial diameter (BAD). The key finding was that long-term in $PM_{2.5}$ exposure (3 μg/m^3) was associated with 0.3% reduction in FMD, an effect equivalent to the effects of active cigarette smoking or 5 years of aging (Brook and Rajagopalan, 2012). This effect was not observed in association with short-term $PM_{2.5}$ exposure, although it should be noted that different exposure assessment methods were used to determine PM concentrations in the short- and long-term exposures. Nitroglycerin-mediated dilatation was not measured, thus precluding determination of a relationship between exposure and vascular smooth muscle function. In a separate study, Briet et al. (2007) found that long-term exposure to PM was associated with an increase in reactive hyperemia, which is a transient increase in blood flow following brief occlusion of an arterial vessel (an ischemic period) in healthy subjects. Furthermore, Zanobetti et al. (2014) demonstrated that short-term exposure to $PM_{2.5}$ was associated with a decrease in BAD, a predictor of cardiovascular risk, in type 2 diabetics.

Recent epidemiological evidence suggests associations between short-term exposure to O_3 and clinical cardiovascular events linked to coronary artery disease, myocardial infarction, and atherosclerosis (Srebot et al., 2009). There is limited evidence suggesting a link with long-term exposure to O_3 (Jerrett et al., 2009; Brook et al., 2010). Although not measuring vascular function, Suissa et al. (2013) demonstrated that low level O_3 exposure was associated with stroke occurrence in a high risk vascular subgroup.

3.3. Evidence from controlled human exposure studies

For an extensive review of clinical evidence linking air pollution exposure to vascular dysfunction, the readers are referred to the review by Moller et al. (2011). Virtually all of the controlled human exposure studies have relied upon forearm blood flow assessments using venous occlusion plethysmography or brachial arterial FMD assessed using ultrasound scanning. Exposure to diesel exhaust (Peretz et al., 2008), ultrafine carbon particles (Shah et al., 2008), and concentrated ambient particulates (CAPs) (Brook et al., 2009) modified vascular function. In the latter study, CAPs exposure inhibited endothelial-dependent vasodilation and promoted vasoconstriction. Mills et al. (2005) found similar effects with whole diesel exhaust (PM plus gases), while diesel exhaust gases alone had no effect. In a separate study, Mills et al. (2008) failed to find an effect of CAPs exposure on FMD. The variability in responses to CAPs was attributed to regional differences in particle chemistry, health status, and/or low particle number (Brook et al., 2010).

Fewer controlled human exposure studies examined vascular function effects of O_3 exposure. In one study, exposure to O_3 in combination with CAPs caused arterial vasoconstriction; the effects of O_3 exposure alone were not examined (Brook et al., 2002). In another study, exposure to O_3 in combination with CAPs increased diastolic blood pressure, while exposure to O_3 alone did not (Fakhri et al., 2009). Exposure to O_3 failed to affect bilateral forearm blood flow in a randomized double-blind crossover study (Barath et al., 2013). These studies were conducted in healthy men, pointing to a need to study impacts of relevant ambient O_3 concentrations in both healthy and susceptible subgroups.

3.4. Evidence from toxicological studies

Toxicological evidence of the effects of air pollution stems from two sources: in vivo vessel assessments in animals infused with vasoactive drugs and ex vivo assessments of isolated vessels or hearts. Sun et al. (2005) demonstrated that long-term CAPs inhalation in atherosclerotic mice decreased vasodilatory responses while increasing vasoconstrictive responses. Others found similar findings with PM, including CAPs (Batalha et al., 2002), diesel exhaust (Knuckles et al., 2008), urban dust (Courtois et al., 2008), and engineered nanoparticles (Cozzi et al., 2006) in different species and strains. In contrast, many studies found PM exposure had little effect on endothelium-independent vasodilation (Moller et al., 2011).

With respect to O_3, three experimental studies that examined vascular function point to impairment. Chuang et al. (2009) found that O_3 exposure significantly modulated vascular tone regulation in mice. Further, vasorelaxation responses in aortas of mice and coronary arteries of rats examined ex vivo were impaired following O_3 exposure (Robertson et al., 2013; Paffett et al., 2015). In one of these studies, the dilation response to an agonist was restored by treatment of vessels ex vivo with antioxidant enzymes and an inhibitor of NADPH oxidase, which generates superoxide anion (Paffett et al., 2015). Furthermore, dilute serum from O_3-exposed rats diminished vasodilatory responses in vessels from naïve rats and exhibited a capacity to scavenge nitric oxide. These results provide additional evidence that air pollutant effects on the vasculature may be due to decreased NO bioavailability.

3.5. Mechanisms underlying endothelial dysfunction

The intricate signaling pathway that mediates endothelium-dependent vasodilatation begins with binding of agonists, such as bradykinin or acetylcholine, to G-protein coupled receptors. Receptor binding leads to calcium influx, which activates endothelial nitric oxide synthase (eNOS). Alternatively, sheer stress due to altered blood flow results in eNOS activation; this response forms the basis for FMD measurements. eNOS in turn generates NO from L-arginine which diffuses to juxtaposed smooth muscle cells and activates soluble guanylyl cyclase. Guanylyl cyclase converts GTP to cGMP, relaxing the smooth muscle via changes in the contractile apparatus (Moller et al., 2011). ED results in a diminished vasodilatory response to agonists or sheer stress.

The principal mechanism by which ED takes place involves the loss of endothelial NO. This reduction may be caused by impaired expression of eNOS, abnormal function of eNOS because of reduced substrate (L-arginine) or cofactor (tetrahydrobiopterin) levels, inadequate activation of the enzyme due to altered cell signaling and/or oxidative destruction of NO by superoxide anion (Rajagopalan et al., 2005). Oxidative stress, commonly found in cardiovascular disease, mainly results from increased activity of NADPH oxidases or xanthine oxidoreductase, leak of electrons from the mitochondria, and/or uncoupled NOS in the vasculature that produces superoxide anion rather than NO (Endemann and Schiffrin, 2004). Multiple disease-related factors contribute to decreases in NO bioavailability, including increased angiotensin II (in the setting of hypertension and chronic renal failure), hyperhomocysteinemia, and impaired insulin regulation in diabetes (Endemann and Schiffrin, 2004).

3.6. AOP: air pollution mixtures and cardiovascular outcomes

PM and O_3 may exacerbate hypertension, atherosclerosis and associated cardiovascular outcomes by contributing to systemic inflammation and vascular oxidative stress. These processes may impact blood vessels and lead to the development of ED. These steps are outlined in the following proposed AOP. As depicted in Fig. 2, inhalation of PM and/or O_3 leads to redox reactions in lung lining fluid and tissue that result in pulmonary inflammation. The leak of inflammatory mediators from the lung into the circulation subsequently triggers systemic inflammation and vascular oxidative stress. A wealth of evidence points to the capacity for systemic inflammation to target systemic arterial vascular endothelium. Macromolecular interactions of inflammatory mediators with endothelial cell receptors and/or other cellular molecules comprise the molecular initiating events in the AOP.

At the cellular response level, multiple pathways may be responsible for reducing NO bioavailability in vascular endothelium. These include decreased eNOS protein levels, decreased eNOS substrate (i.e., L-arginine) or cofactor (e.g., tetrahydrobiopterin) levels, decreased eNOS activation due to altered cell signaling and increased destruction of NO by superoxide anion.

Cellular responses may lead to organ responses. A reduction in NO bioavailability may result in ED. Under normal physiological conditions, the endothelium mediates vascular dilation to maintain normal blood pressure and ensure adequate organ perfusion, prevents platelet activation and clot formation, and inhibits leukocyte adhesion. While ED's impact will depend on which local vascular bed is affected, net effects may lead to vasoconstriction, increases in adhesion molecules and associated inflammation, platelet aggregation, coagulation, thrombosis, and smooth muscle proliferation.

These systemic vascular responses have physiological consequences that can be measured clinically. These include increased blood pressure stemming from increased vascular resistance and/or remodeling, increased plaque/atheroma size from thrombotic and inflammatory mechanisms leading to atherosclerotic progression, and, when present in coronary circulation, coronary vasospasm, which predisposes to myocardial ischemia and increases risk of myocardial infarction.

Finally, clinical consequences at the individual level may lead to effects at the macro level, which affect the population as a whole. Such effects, whether acute or chronic, may aggravate preexisting cardiovascular disease and as such increase cardiovascular morbidity and mortality. Outcomes may be reflected at the population level by increased numbers of emergency department visits and hospital admissions.

4. Discussion and conclusions

This work describes a conceptual model for air pollution mixtures that links initiating events at the cellular and molecular level to population-wide impacts. This approach incorporates information from observational and experimental studies into a sequence of steps occurring over multiple levels of biological organization. It also provides a framework for converging the effects of air pollutants based on common underlying mechanisms. The examples presented here, AHR and ED, are primary mechanisms for respiratory and cardiovascular dysfunction, respectively. They are both physiologic changes at the organ level and measurable endpoints that serve to integrate the effects of individual criteria air pollutants found in inhaled mixtures.

This approach to multipollutant air pollution mixtures provides a platform to identify what is known and unknown, prioritize areas of research, and initiate targeted research efforts in an expedited and cost-effective manner. Furthermore, this framework allows the incorporation of molecular or cellular biomarker data that is predictive of clinically significant endpoints, as it becomes available in the future. Along these lines, epidemiologic and/or controlled human exposure studies examining the relationship between exposure to multiple air pollutants and measures of AHR and ED may help fill the gaps in knowledge and better lay a foundation for applying this conceptual model to the regulatory setting. Additional toxicological studies evaluating the effects of multiple air pollutants on upstream events such as allergic sensitization (in the case of AHR) or NO bioavailability (in the case of ED) may provide biomarker data relevant to these clinical endpoints. An important limitation of this approach is the fact that population effects from air pollution exposure described above may be mediated by mechanisms other than the ones focused on here. For example, increased cardiovascular morbidity may be due to autonomic effects of air pollution, rather than to systemic inflammation and oxidative stress, which were the focus of the second case report. A second limitation of this approach is that it does not address dose–response considerations—either in terms of the dose of a given air pollutant required to achieve an adverse

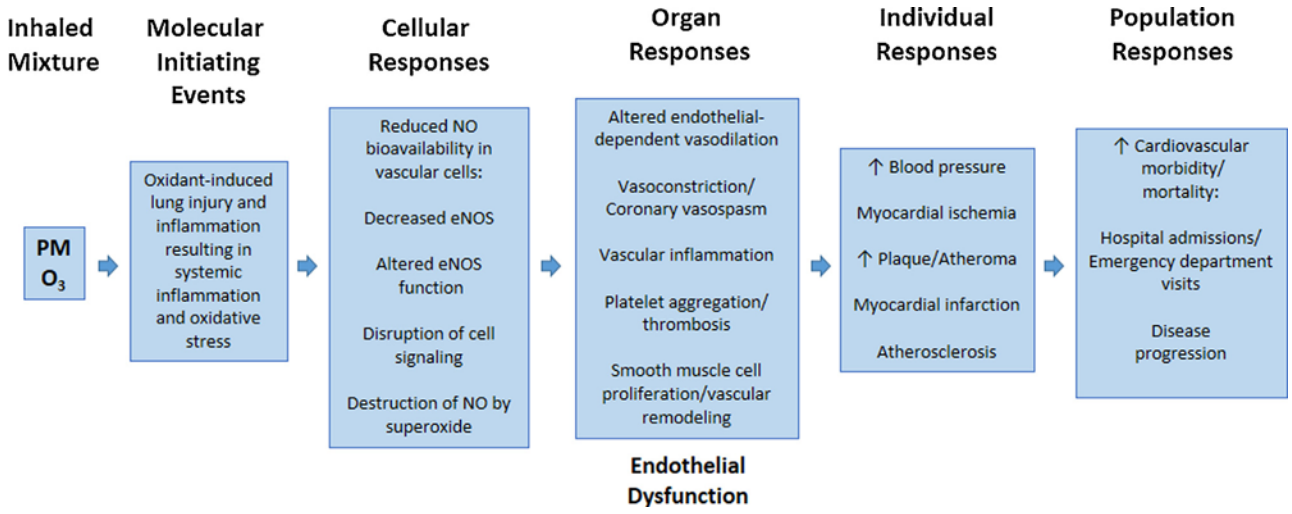

Fig. 2. AOP: air pollution mixtures and cardiovascular outcomes.
This AOP depicts a sequential series of higher order effects linking exposure to PM and O_3 to an adverse outcome with relevance to risk assessment, i.e., endothelial dysfunction. Cellular responses refer to responses in all vascular cell types. NO: nitric oxide; eNOS: endothelial nitric oxide synthase.

effect or in terms of the relative potency of chemicals in a given mixture. Furthermore toxicokinetic and toxicodynamic data have not been incorporated into the model. Finally, this approach does not account for cellular/tissue adaptation and repair, which are normal homeostatic responses likely to have their greatest impacts with low dose exposures.

Despite these limitations, the proposed AOP framework may be a useful strategy to streamline hazard assessment of air pollution mixtures because it shifts focus away from the complexity of air pollution mixtures to the capacity for that mixture to impact a common clinical endpoint that is measurable in humans. This approach complements existing and emerging statistical, modeling, and high throughput methods that seek to achieve dimension reduction in multipollutant risk assessment. Finally this conceptual model may help move us one step closer to explicit consideration of multipollutant evidence in standard setting, which represents a significant and needed paradigm shift.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

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