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The role of oxidative stress and innate immunity in O₃ and endotoxin-induced human allergic airway disease

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Summary: Ozone (O₃) and endotoxin are common environmental contaminants that cause asthma exacerbation. These pollutants have similar phenotype response characteristics, including induction of neutrophilic inflammation, changes in airway macrophage immunophenotypes, and ability to enhance response to inhaled allergen. Evoked phenotyping studies of volunteers exposed to O₃ and endotoxin were used to identify the response characteristics of volunteers to these pollutants. New studies support the hypotheses that similar innate immune and oxidant processes modulate response to these agents. These include TLR4 and inflammasome-mediated signaling and cytokine production. Innate immune responses are also impacted by oxidative stress. It is likely that continued discovery of common molecular processes which modulate response to these pollutants will occur. Understanding the pathways that modulate response to pollutants will also allow for discovery of genetic and epigenetic factors that regulate response to these pollutants and determine risk of disease exacerbation. Additionally, defining the mechanisms of response will allow rational selection of interventions to examine. Interventions focused on inhibition of Toll-like receptor 4 and inflammasome represent promising new approaches to preventing pollutant-induced asthma exacerbations. Such interventions include specific inhibitors of innate immunity and antioxidant therapies designed to counter the effects of pollutants on cell signaling.

Keywords: ozone, endotoxin, allergic asthma

Introduction

Asthma and respiratory tract disease morbidity (emergency room visits, hospitalizations, and rescue medication use) are clearly associated with exposure to increased levels of ambient air ozone (O₃) (1). A notable risk population for O₃-induced exacerbation of respiratory illness is asthmatic children (1, 2). Multiple reports from Atlanta (3), Los Angeles, Mexico City (4–6), and New England (7) have demonstrated that levels of O₃ below the current 1 and 8 h O₃ standard (0.12 and 0.075 parts per million, respectively) are linked with asthma exacerbation. Respirable particulate matter (<10 micron in size) is also associated with episodes of increased asthma

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exacerbation (8). Increased ambient air particulate levels have been linked to need for asthma medication in a cohort of asthmatics studied in Utah (9, 10). Hospitalization due to increased asthma severity in Seattle was found to occur in conjunction with increases in airborne particulate matter (11).

Similar observations have been made in Germany, the Czech Republic, and Mexico City, and other locations (12–14). Endotoxin present in airborne particles is one of the most significant causes of acute disease in persons with established disease (15–17). Endotoxin has been identified on both PM_{2.5} and PM₁₀ particles (18, 19) and is a notable problem in occupational settings and domestic environments (20, 21). It has been observed that disease severity of mite-sensitive asthmatics correlated better with indoor endotoxin levels than with levels of mite allergens (22–24). Though environmental endotoxin is perhaps best known in the context of the hygiene hypothesis (in which exposures to endotoxin in early life are thought to prevent development of allergic disease), many studies reveal complex gene–environment interactions that suggest that genetic factors may increase risk for developing disease after endotoxin exposure (25–27). Taken together, the epidemiological data indicate that endotoxin and O₃ are important environmental triggers for asthma (17, 24, 28–32).

This review examines the impact of O₃, one of the most commonly encountered pollutants in the United States, and endotoxin, a component of bioaerosols and particulate air pollutants, on airway responses in healthy and allergic persons. Endotoxin is a well-studied pathogen-associated molecular pattern (PAMP) stimulant of the innate immune system, exerting its effects via ligation with the Toll-like receptor 4 (TLR4) on macrophage and monocytes membranes. By comparing and contrasting responses of endotoxin to O₃, it is possible to gain insight into the mechanisms which may regulate response to pollutants. Air pollutant exposure has also been shown to directly augment response to second agents, such as allergen.

In addition to examining innate immune mechanisms, this review comments on the role of oxidative stress in regulating response to pollutants. Oxidant stress is an important element in the pathophysiology of number of lung diseases, especially with exacerbation associated with pollutant exposure (33) (Fig. 1). Genetically defined deficiencies in antioxidant genes have been identified as risk factors for increased response to pollutants, such as O₃ and lipopolysaccharide (LPS). Interventions focused on enhancing antioxidant capabilities in persons at risk for pollutant induced disease represent a novel approach to prevention of asthma exacerbation. Finally,

potential interventions to mitigate the impact of pollutants on airway function based on mechanistic understanding of the actions of pollutants on the airway are discussed.

Evoked phenotype response to pollutants in volunteers

Epidemiological studies provide important insights into the impact that pollutants have on disease, populations at risk for adverse health effects due to pollutants, and potential genetic factors which may impact pollutant induced disease. Animal studies are essential in defining mechanisms by which environmental agents affect a number of responses in intact organisms, and *in vitro* studies with isolated cells can identify molecular mechanisms by which pollutants modulate disease. However, to understand the processes by which pollutants modify physiological responses in humans, it is necessary to focus on a cohort of people exposed to ambient pollutants with careful assessments of physiologic outcomes, or expose volunteers to experimentally derived pollutant atmospheres, usually employing either pollutant chambers or application of pollutant materials onto an airway surface in a nasal or inhalational challenge. Our group has focused extensively on such studies and has employed these approaches to define a variety of pollutant evoked phenotypes in humans.

O₃ effects on lung function and inflammation in healthy volunteers

Controlled exposure studies have consistently revealed three airway response phenotypes to O₃ in humans. The most extensively studied is a pain mediated nociceptive decrease in FVC and FEV₁, accompanied by chest discomfort when deep inspiration. Nociceptive changes in lung function are observed as quickly as 1 h after exposure but may persist up to 24 h after exposure (34, 35). Another response phenotype is change in airway reactivity. This is variably reported and may occur more avidly in asthmatics. The third response phenotype is development of airway inflammation, defined primarily by an influx in granulocytes.

Despite the temporal relationship between inflammatory and spirometric responses to acute O₃ challenge, these changes do not correlate with each other. In some cases, a robust inflammatory response may be seen in an individual with little change in lung function. Conversely, persons with a notable nociceptive effect on lung function may experience little or no increase in airway polymorphonuclear neutrophils (PMNs). Together, these results indicate that separate mechanisms mediate these responses.

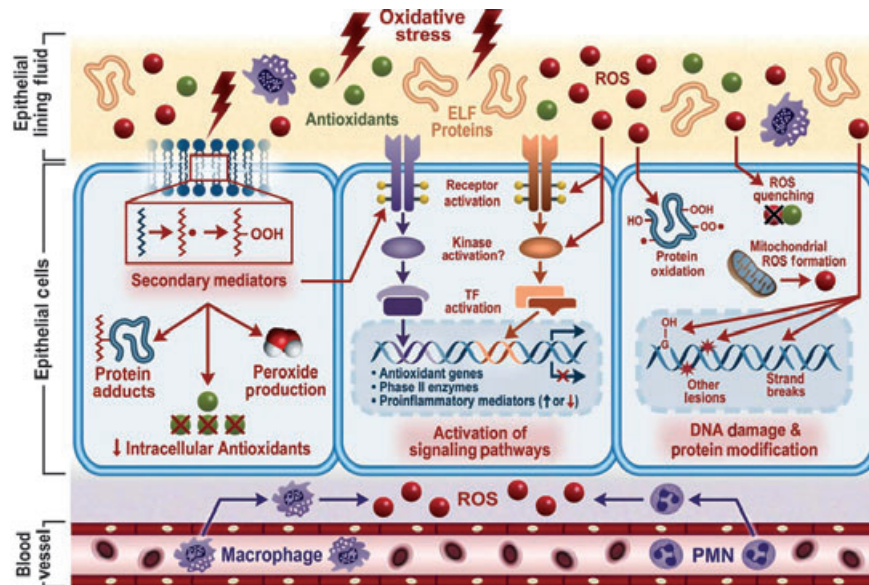


Fig. 1. Mechanisms involved in oxidant pollutant-induced adverse health effects. Oxidant pollutants may elicit their effects through (1) the production of secondary mediators generated by reaction of pollutants or pollutant-induced ROSs or free radicals with lipids in the ELF or cell membrane as well as proteins and antioxidants; (2) activation of signaling pathways by ROSs or secondary mediators; (3) oxidation of cellular proteins; (4) damage to DNA. In addition to pollutant-induced generation of ROSs, endogenous sources of ROSs such as inflammatory cells, phagocytes recruited to the site of injury, and other cellular processes may contribute to the oxidative stress state caused by pollutant exposure. Antioxidant molecules and enzymes mitigate the effects of ROSs in the body. TF, transcription factor; PMN, polymorphonuclear leukocyte. From Ref. (33), with permission.

In either controlled exposure settings or with ambient exposures, the effective dose of O₃ is influenced by the atmospheric concentration of O₃, the degree of minute ventilation (essentially the amount of O₃ containing air is delivered to the airspace in a minute), and the duration of exposure. Most experimental protocols manipulate minute ventilation with exercise. Without exercise to increase minute ventilation to >8 l/min, little effect of O₃ on lung function can be observed at levels below 0.50 ppm. However, as minute ventilation increases with exercise, even modest levels of ambient O₃ experience will induce decreases in FEV₁, FVC, airway resistance, and increased chest symptoms (36, 37). Models have been developed based on nociceptive decreases in lung function which predict the responses associated with time (T) × concentration (C) × ventilation factors.

The effect of O₃ can be seen even with very low levels. The current National Ambient Air Quality Standard (NAAQS) for O₃ is 0.075 ppm averaged across 8 h. However, epidemiologic studies focused on health outcomes have noted increased asthma events at levels <0.06 ppm. We have examined the effect of experimental exposure to 6.6 h exposure to 0.06 ppm O₃ with moderate exercise and have found nociceptive decreases in lung function and increased PMNs in airway sputum. These observations have led to recommendations that exercise be minimized in persons who are susceptible to the effect of O₃.

As noted above, the best studied inflammatory response to O₃ in humans is influx of neutrophils to the airway, with bronchoalveolar lavage, mucosal biopsy, and sputum induced samples all demonstrating that O₃ exposure cause influx of PMNs into the airway, at levels ranging from 0.06 to 0.4 ppm. Neutrophil influx occurs early (1–6 h after exposure) and can persist until the next day (2). Inflammatory mediators such as interleukin-1 β (IL-1 β), IL-6, IL-8, prostaglandin E₂ (PGE₂), lymphotoxin B₄ (LTB₄), TXB₂, tumor necrosis factor α (TNF α), fibronectin, plasminogen activator, and elastase are also increased by O₃. Direct comparison of the kinetics of mediator response to O₃ shows that changes in IL-6 and PGE₂ peak 1 h after exposure, whereas fibronectin and plasminogen activator are higher 18 h after exposure (38–40).

Many people are exposed to increased ambient O₃ on multiple consecutive days, especially in the summer months. Multi-day exposure studies reveal that an initial enhancement of the nociceptive effect on lung function, followed by an adaptation of this response (41). Additionally, one group reports that repeated exposures causes an adaptation to the neutrophil response to O₃ (42). A second study revealed that repeated exposures caused an influx on macrophages to the airway (with no change in PMNs), suggesting a role for macrophages in regulating innate immune responses to O₃ (43).

We have employed flow cytometry to immunophenotype monocytes and macrophages recovered from sputum as well as to assess phagocytic function and oxidative burst of these cells. After O₃ challenge, we typically see decreased numbers of macrophages in airway sputum. However, we also see increased expression of innate immune surface proteins (CD11b, CD14, TLR4), antigen presentation markers (CD80, CD86, HLA-DR), and immunoglobulin receptors (CD16, CD23, and FcεRI) (44–48). We also observe that induced oxidative burst and phagocytic function are increased in these studies. Taken together, these observations suggest that O₃ exposure may enhance response to other biological agents (49). Additionally, we have found that hyaluronic acid, a ligand for TLR4 and CD44, is increased in airway sputum after O₃ challenge (46). Hyaluronic acid activates airway macrophages, resulting in an initial innate immune response that results in airway neutrophilia. Overall, it seems likely that monocytes and macrophages may play a central role in mediating the effects of this pollutant, and may be much more important with repeated exposures.

O₃ effects on lung function and inflammation in allergic asthmatic volunteers

Compared to healthy volunteers, persons with asthma may have enhanced response to the effect of O₃. There are mixed reports suggesting that asthmatics are more susceptible to the nociceptive decreases in lung function caused by O₃ (50). It has also been reported that asthmatics have enhanced neutrophil responses to O₃ in controlled exposure studies (51, 52). However, other investigators do not report such differences. These discrepancies may be due to subtle differences in the recruitment and study entrance criteria for asthmatics. Moderate and severe asthmatics, which might be expected to have enhanced responses to O₃, are rarely recruited to O₃ challenge studies. However, mild intermittent asthmatics may differ sufficiently from mild persistent asthmatics to account for these differences in reported PMN responses to O₃.

Other aspects of the inflammatory response to O₃ have also been examined in allergic asthmatic volunteers. Eosinophilic response in the nasal and bronchial airways has been observed after O₃ challenge (53, 54). Additionally, asthmatics have been found to have increased IL-1β, IL-6, and decreased IL-10 in airway sputum after O₃ challenge (46). It has been observed in a study of IL-10 knockout mice that IL-10 protects against the inflammatory effect of O₃ (55). As noted previously, macrophage and monocytes populations have increased expression of innate immune, antigen presentation and

immunoglobulin receptors. We have found that macrophages recovered from sputum of asthmatics after O₃ challenge have increased expression of CD23, FcεRI and TLR4 compared to normal volunteers (46). Thus, while PMN response to O₃ may not be remarkably different between asthmatic and non-asthmatic volunteers, asthmatics likely have unique innate and acquired immune responses which impact asthma pathobiology.

An important element of O₃ in asthma is enhancement of response to allergen in allergic volunteers. Epidemiological studies demonstrate that exacerbations of asthma occur 24–48 h after increases in ambient air O₃ occur, consistent with the idea that O₃ primes the airway for increased response to second agents, such as allergen. The changes in macrophage surface markers following O₃ challenge noted above are consistent with increasing antigen presentation and IgE recognition of allergen in the airway.

Challenge to 0.12 ppm for 1 h has been reported to increase immediate response to inhaled allergen (56). While other studies of low levels of O₃ have either not uncovered an increased response to allergen, there are trends for increased response in subsets of volunteers, suggesting genetic or other factors may modulate O₃ enhancement of allergen response in these subsets (57). Consecutive multi-day challenge with levels of O₃ as low as 0.125 ppm enhances response to inhaled allergen (58). Furthermore, relatively short exposure protocols using 0.16 and 0.25 ppm O₃ with exercise clearly demonstrate increased response to inhaled allergen (59, 60). Using a nasal challenge protocol, we observed that eosinophilic late phase response to allergen is enhanced following challenge with 0.4 ppm O₃ (61). Others have shown that challenge with 0.27 ppm O₃ after inhaled allergen challenge also augments late phase eosinophilic but not neutrophilic response to allergen (62).

Evoked phenotype responses to endotoxin in healthy volunteers

Endotoxin has been identified on both PM_{2.5} and PM₁₀ particles (60–62) and has been associated with increased airway inflammation in human challenge studies (31). Environmental endotoxin is perhaps best known in the context of the hygiene hypothesis, in which exposures to endotoxin in early life have been linked to decreased development of allergic disease and asthma. However, environmental endotoxin is also clearly linked to respiratory tract morbidity as well. Endotoxin rich PM found in agricultural settings has been linked to occupational lung disease and ambient air endotoxin is associated

with increased respiratory tract disease in allergic and non-allergic persons (15, 16).

Inhalational challenge with endotoxin doses ranging from 0.5 to 60 µg has been employed to assess the effect of this agent on airway responses (63–67). Higher doses (typically above 5 µg) can induce decreases in lung function, increased airway inflammation and systemic inflammation. Recently, the time course of the inflammatory response was assessed following challenge of eight volunteers with 20 µg of endotoxin (68). Sputum was collected at baseline, 6 and 24 h and 7 days after challenge. At 6 h, there is an increase in lymphocytes and neutrophils. At 24 h, there was an increase in total cells, macrophages, neutrophils, and lymphocytes compared to baseline values. Thus, endotoxin challenge causes transient inflammation which is evident 6 h after challenge, peaking at 24 h with resolution by 7 days.

Perhaps more important is the effect of endotoxin inhalation on airway macrophage and monocyte populations. We examined the effect of endotoxin doses of 2500, 5000, and 10 000 endotoxin units (EU) on airway inflammatory cells (64). We did not observe significant increases in total numbers of PMNs. However we did observe enhanced monocytes and macrophage phagocytosis, increased expression of surface CD11b and mCD14 on monocytes and neutrophils, and increased IL-13 levels, and γ-IFN levels were significantly decreased. A 5-µg (or approximately 20 000 EU) dose of endotoxin reliably evokes airway inflammation, change in airway cytokines, increases in circulating PMNs and may enhance airway reactivity in volunteers, though changes in symptoms (fever, malaise) and lung function are not generally seen with this dose. At this dose, we also observed increased expression of CD80, CD86, HLA-DR on macrophages, decreased phagocytic function of monocytes and macrophages, and influx of a discrete population of dendritic cells into airway sputum. We also observed increased levels of IL-1β and granulocyte macrophage colony-stimulating factor (GM-CSF) (69, 70).

We have also examined the effect of recovered ambient air coarse particulates on airway inflammation and macrophage response in nine healthy volunteers (18). These volunteers underwent challenge to saline, PM_{2.5-10} recovered from ambient air that was heated to inactivate biological material, or non-heated PM on three separate occasions. Inhaled non-heated PM induced airway PMNs influx and macrophage mRNA TNFα, increased eotaxin, and increased expression of CD11b, CD14, and HLA-DR on sputum macrophages. Heated PM also induced PMNs but did not result in increased macrophage mRNA TNFα, cell surface markers or phagocytosis.

These findings with ambient PM support the use of 20 000 EU endotoxin challenge as a model of response to environmental bioaerosols.

Animal studies have also suggested that tolerance to repeated exposure to inhaled endotoxin may occur. We examined the effect of repeated nasal challenge with endotoxin for three consecutive days in normal volunteers and found that there was an attenuation of PMN response to endotoxin by the third day (71). It is not known if repeated challenge with endotoxin causes decreased PMN response in the lower airway or which mechanisms account for this.

Evoked phenotype responses to endotoxin in allergic asthmatic volunteers

It has been shown that allergen-induced inflammation enhances expression of molecules which mediate response to endotoxin (most notably CD14 expression on airway monocytes), indicating that allergic individuals may have enhanced response to environmental endotoxin (68, 69). We have shown that response to endotoxin correlates with macrophage expression of CD14 and that macrophage CD14 correlates with sputum eosinophils in allergic asthmatic volunteers. We have also shown that endotoxin challenge of the nasal airway results in eosinophils influx in allergic asthmatic volunteers (72). Michel *et al.* (73, 74) have reported that asthmatics have increased airway reactivity following inhalation of 20 µg endotoxin compared to normal volunteers. They also examined responses to 0.5, 5, and 50 µg doses of endotoxin in 15 volunteers and assessed responses in persons stratified on the basis of fever and airway reactivity changes due to endotoxin (75). The seven people with increased temperature also had increased circulating C-reactive protein (CRP) and PMNs. Those with increased airway reactivity had increased airway eosinophil cationic protein (ECP) in sputum. The degree of systemic response to endotoxin was inversely related to atopy status. Together, these observations suggest that asthmatics have enhanced effect of endotoxin in the airway but muted systemic response.

As with O₃, endotoxin enhances response to inhaled allergen. We have found that challenge of the nasal airways of allergic asthmatics with a subthreshold dose of endotoxin after subthreshold dose of mite allergen resulted in enhanced eosinophils and neutrophil influx (76). This suggested that endotoxin could amplify the allergen induced response in the airway. We have also examined the response to inhaled allergen in persons exposed to particle bound endotoxin equivalent to that observed in the 'sick-building syndrome' and observed increased response to mite allergen in mite allergic

volunteers (77). Schaumann *et al.* (78) recruited 17 allergic asthmatic volunteers to undertake segmental challenge with allergen, endotoxin, and the combination of the two. They found that endotoxin enhanced cellular response to allergen, with increases in lymphocytes, neutrophils, eosinophils, monocytes, and myeloid dendritic cells (mDCs) as well as LPS-binding protein, IL-1 α , IL-6, and TNF α . Most notably, it was found that mDCs had enhanced antigen presenting activity as noted in assays of T-cell proliferation induced by the mDCs. Consistent with this observation, we have observed that airway macrophages have enhanced Fc ϵ R1 receptors after challenge with 10 000 EU (low dose) endotoxin, suggesting that response to allergen might be enhanced after endotoxin challenge (79). Taken together, these observations support the hypothesis that endotoxin enhances allergen induced response in the airway, perhaps via both IgE-dependent mechanisms and via presentation of antigen to mucosal T lymphocytes.

Use of evoked phenotypes to explore shared mechanisms of response to endotoxin and O₃

As endotoxin and O₃ both cause airway inflammation, changes in macrophage and dendritic cell populations, changes in macrophage function, and enhance response to allergen, it seems likely that common factors influence response to these pollutants. To examine the correlations between response to O₃ and endotoxin, we performed inhalation challenge with 15 volunteers to 0.4 ppm O₃ and 20 000 EU of endotoxin (47). We noted a positive correlation between O₃- and endotoxin-induced sputum neutrophilia ($r = 0.73$, $P = 0.007$).

Because O₃ is a direct oxidant and LPS can induce oxidative burst and generation of reactive oxygen species (ROS), we also hypothesized that GSTM1 would modulate inflammatory responses to both agents (80, 81). Thirty-five of the 37 volunteers who underwent O₃ challenge were genotyped for GSTM1, with 19 volunteers being sufficient in GSTM1 (have a genotype associated with producing GSTM1 at the protein level), and 16 had the GSTM1 null genotype (resulting in no GSTM1 production) (80). We examined the immediate effect of 2 h, 0.4 ppm O₃ exposure on lung function, as well as inflammatory endpoints present 4 h and 24 h after challenge. We found that GSTM1 null volunteers did not have differential susceptibility to the effect of O₃ on lung function 4 or 24 h post exposure, or a significant effect on PMN influx 4 h after challenge. However, 24 h after challenge, GSTM1 null volunteers had significantly increased neutrophil and macrophage

numbers in airway sputum (Fig. 2). This timepoint is consistent with numerous epidemiological observations that suggest that exacerbations of asthma and other adverse effects of O₃ occur 24–48 h after the exposure occurs.

We also examined the response to endotoxin in 35 normal volunteers as reflected in sputum recovered 4 h after inhalation challenge (81). We again stratified inflammatory response on the basis of GSTM1 null genotype and found a notably increased sputum PMN response to CCRE in the GSTM1 null volunteers. Taken together, the results of the O₃ and LPS studies reveal that GSTM1 modulates response to both agents, indicating that oxidative stress modulation plays a role

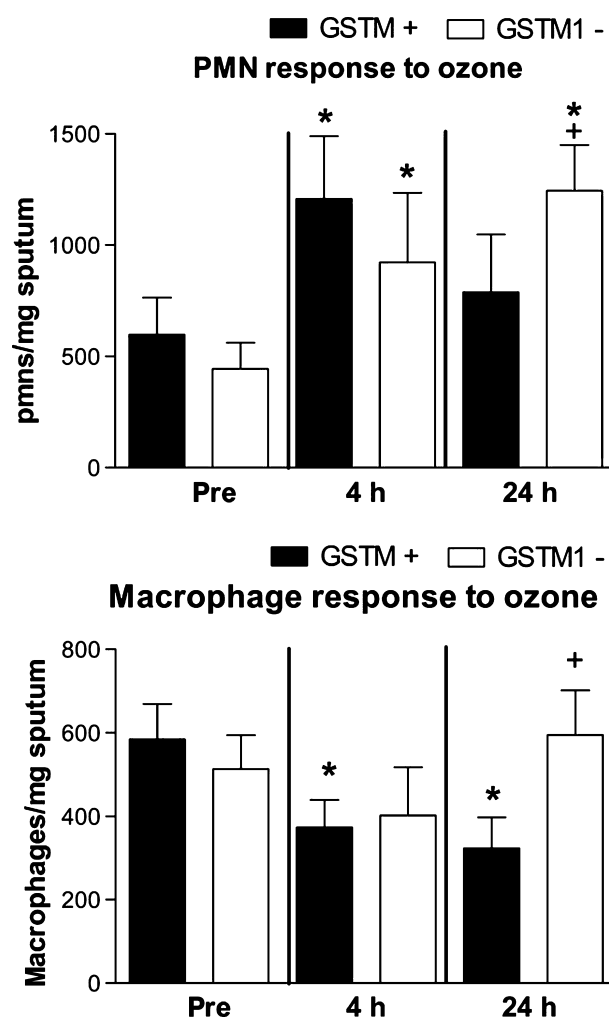


Fig. 2. Effect of GSTM1 genotypes on PMN and macrophage numbers in sputum in response to ozone. Mean (SEM) PMNs per milligram of sputum (top) and macrophages per milligram of sputum (bottom) before (baseline) and 4 and 24 h after ozone exposure in GSTM1+ ($n = 19$, solid bars) and GSTM1- ($n = 16$, open bars) subjects. * $P < 0.05$ versus baseline. + $P < 0.05$, GSTM1+ versus GSTM1-. From Ref. (80), with permission

in response to both agents. Overall these data in human volunteers supports the role for both innate immune processes and oxidative stress biology in modulating and mediating response to pollutants.

Innate immunity and inflammatory response to pollutants

TLR4 and response to pollutants

While there is substantial variation in the physical and chemical nature of a number of ambient air pollutants including O₃ and particulate matter (PM), each of these agents induces neutrophilic inflammation of the airway. As neutrophil influx is a common feature of innate immune activation, this suggests that innate immune mechanisms represent a common pathway by which inflammatory responses to pollutants is mediated. It is well known that the TLR4 mediates LPS signaling, and the similarities between inflammatory response to O₃ and LPS have supported the hypothesis that TLR4 may play a role in mediating response to O₃ as well.

Among the initial reports that focused on the role of TLR4 on response to O₃ are those by Kleeberger *et al.* (82), who compared responses to O₃ between and C3H/HeOuJ (OuJ) mice, which differ only at a missense mutation in the third exon of the *Tlr4* gene. They found that permeability of the airway as well as message for TNF α was decreased in the HeJ strain which has decreased TLR4 mediated responsiveness. Using the same murine model, this group also reported that TLR4 modulated iNOS expression and that subsequent modulation of NO was important in innate immune regulation of O₃ induced responses (83). TLR4 also modulates inflammatory response to residual oil fly ash (ROFA) as well.

Hollingsworth *et al.* (84) compared response of genetically modified, TLR4-deficient mice [C57BL/6(TLR4^{-/-})] to residual oil fly ash, LPS, and O₃. They found that TLR4^{-/-} mice essentially unresponsive to LPS. In contrast, TLR4^{-/-} and wildtype mice did not differ in their inflammatory responses to ROFA or O₃, as characterized by neutrophil, cytokine, and protein content of recovered bronchoalveolar lavage fluid. However, TLR4^{-/-} mice did not develop increased airway responsiveness unlike the wildtype mice. Thus, there were specific response phenotypes that are impacted by deletion of TLR4. These studies by both of these groups demonstrate a role for TLR4 in response to pollutants, including O₃.

However, despite strong mechanistic data that suggest that TLR4 mediates response to O₃, the question of how this oxidant gas activates TLR4 remained unclear. It had been speculated by many groups that O₃ induced injury of airway

cells resulted in release of second messengers or damage-associated molecular pattern (DAMP) mediators. Garantziotis *et al.* (85) hypothesized that hyaluronic acid (HA), a DAMP known to mediate non-infectious lung injury, might account for increased response to O₃. They found that mice challenged with O₃ had increased HA in BAL fluid. They also found that animals deficient in CD44, the primary receptor for HA, had decreased inflammatory response and decreased airway reactivity compared to wildtype mice, despite having similar increases in airway HA. They also found that animals deficient in inter- α -trypsin inhibitor (which facilitates hyaluronan binding) also had decreased response to O₃, despite increased levels of HA. Furthermore, overexpression of HA resulted in increased response to O₃. This is an initial example of an O₃-induced DAMP playing a role in O₃-induced inflammation and airway reactivity.

These investigators then explored if TLR4 may also mediate response to O₃ via increases in HA (86). They found that TLR4-deficient and wildtype mice had similar degrees of cellular inflammation, injury, and HA levels following O₃ challenge. However, TLR4^{-/-} mice had decreased reactivity responses to both O₃ and HA than did wildtype mice. TLR4-dependent cytokine profiles were similar between O₃ and HA challenge and *in vitro* challenge of bone marrow-derived macrophages to O₃ reveals a TLR4-dependent pattern of NF- κ B activation and cytokine production. These data show that HA effects are also mediated, at least in part, by TLR4.

As many persons are exposed to more than one agent, the effect of O₃ on response to other agents is also important. To address this question, the effect of O₃ on subsequent response to LPS was examined in murine models (87). Mice sensitive to O₃ were exposed to air or O₃ and then challenged to LPS. Animals pre-exposed to O₃ had increased airway reactivity, cytokine and protein levels in BAL, increased evidence of LPS-induced signaling in lung tissue, and increased serum IL-6. However, there was decreased airway cells in animals exposed to both O₃ and LPS. This was likely due to active apoptosis, as O₃ also was associated with increased TLR4 expression on lung cells and increased functional response of cells to LPS.

Given the effect of O₃ on the production of HA and the interaction of HA with both CD44 and TLR4, the role of HA in mediating the effect of O₃ on LPS response was also examined (88). O₃ was again shown to enhance LPS-induced inflammation, cytokine production, and airway reactivity. HA yielded a similar result. Furthermore, HA treatment of bone-marrow derived macrophages resulted in TLR4 peripheralization and lipid raft formation. Overall, these data support the idea that O₃ (in part via production of HA) may enhance

macrophage and other cell response to second agents by increasing expression of TLR4, resulting in enhanced response to LPS and similar agents.

Inflammasomes and environmentally induced inflammation

Another innate immune response mechanism that may mediate response to pollutants is the inflammasome. Inflammasome complexes are associated with production of proforms of IL-1 β , IL-18, and IL-33 (89, 90). Subsequent activation of caspase-1 by a variety of stimuli results in cleaving of the pro-forms of cytokines to result in secretion of active cytokines into tissue spaces. Ting *et al.* from our group have focused on the role of NLRP3 inflammasomes on host defense to a number of pathogens, including influenza (91), staphylococcus (92), gonococcus (93), and mycobacterium tuberculosis (94), showing that NLRP3 is central in the response to each of these agents.

In addition to responses from pathogens, NLRP3 may also mediate response to exogenous or environmental agents. Ting *et al.* (96) have shown that NLRP3 mediated response to alum (95) as well as the effect of lipids on insulin responsiveness. These are relevant, as alum is a model exogenous particle, and we and others have shown that environmental exposure to PM can modify serum lipid levels (97). Thus, just as TLR4 has an important role in mediating response to pollutants, it is likely that inflammasomes play an important role in this response.

As noted above, inflammasome-mediated cytokine responses involve production of IL-1 β , IL-18, and IL-33 proforms with second signals resulting in active cytokine release. LPS is known to promote pro-IL-1 β , and adenosine triphosphate (ATP) is known to activate caspase-1 and cytokine release via interactions with the P2X7 receptor. LPS is an important component of ambient air PM and other environmentally encountered bioaerosols. ATP is known to be secreted by epithelial cells exposed to O₃ *in vitro*. Other environmental agents or DAMPs (that are produced following pollutant exposure), which activate inflammasomes include asbestos, silica, UV radiation, alloy particles, hyaluronic acid, urate, and cholesterol (98). Taken together, these findings suggest that both TLR4 and inflammasomes regulate response to a variety of exogenous pollutants and that innate immune processes are central to inflammatory response to pollutants.

Oxidative stress and pollutant-induced inflammation

Oxidative stress and airway inflammation

There is a growing body of evidence that oxidant/antioxidant biology is an essential element in airway inflammation resulting from exposure to air pollutants (reviewed in 99).

ROS can derive from a number of sources, including O₃, quinone species generated by the metabolism of polyaromatic hydrocarbons (PAHs) from diesel exhaust (DE), tobacco and wood smoke, and Fenton chemistry involving metals. Additionally, endogenous cellular oxidants are produced by granulocytes, macrophages and epithelial cells in response to pathogens and PM (including biological elements found in PM like LPS). These oxidants include ROS and nitrogen species produced by NADPH oxidase and iNOS as reviewed in Ref. (100). ROS and reactive nitrogen species (RNS) have long been known to participate in inflammation by directly causing tissue injury.

It is increasingly appreciated that these molecules are also key elements of signal transduction which regulate inflammatory responses by cells to a number of agents, as reviewed in Ref. (101). Exogenous hydrogen peroxide has been shown to activate NF- κ B in both T lymphocytes and epithelial cells via actions on signaling molecules SHIP-1 and p56lck (T cells) and the tyrosine kinase Src (epithelial cells). IL-1 β signaling of activation of NF- κ B requires oxidants generated by NADPH oxidase 2 (Nox2), and TLR4 signaling of NF- κ B activation in response to LPS involves activation of Nox4. Generation of endogenous oxidants has recently shown to play an important role in ATP-induced IL-1 β secretion (102). Endogenous oxidants may also play a role in mast cell responses following cross-linking of IgE (103) as well as having an important role in the actions of cyclooxygenase and lipoxygenase, which catalyze oxidative production of eicosanoid mediators of inflammation (104–108).

Antioxidant enzymes and genetic variability in environmental lung disease

Response to oxidant stress involves production of a number of antioxidant response element (ARE) phase II enzymes such as NQO1, GSTM1, GSTP1, HO-1, and others (109–112). Expression of these enzymes is regulated by the transcription factor NRF2 that translocates to the nucleus after oxidative attack on KEAP1, which binds NRF2 in the cytoplasm (113). The importance of NRF2 is highlighted by a series of studies in which lung injury due to exposure to allergen (IgE mediated), viral infection, LPS (CD14/TLR4), bleomycin, hyperoxia, and O₃ are all markedly enhanced using NRF2 knockout animals (114–119). Furthermore, epidemiological, animal and human challenge studies demonstrate that defects in NRF2-modulated proteins (GSTM1, NQO1, and selenoproteins like GPX1-3) are associated with environmental asthma, viral injury of the lung and response to PAHs and O₃ (114, 115,

120). The observations that LPS signaling involves oxidant-associated signal transduction and that O₃ exerts oxidant stress in epithelial cells and monocytes further support the hypothesis that oxidant stress plays a central role in acute environmental asthma.

Supporting the idea that genetic variability may modify response to pollutants or antioxidants are a number of reports on glutathione S transferase M1 (GSTM1), a member of the μ family of GSTs regulated by NRF2. GSTM1 has a null allele (GSTM1*0) which results in no protein expression in approximately 40% of the population. Another GST of interest is GSTP1, in which single nucleotide polymorphisms (SNP) at the 105 position are important regulators of susceptibility to oxidant stress. Glutathione-S-transferase genes play a role in lung growth, as shown in a study of a cohort of 1954 school-children in southern California, in which children with the GSTM1 null allele or a GSTP1 val105/val105 genotype have decreased lung function growth over a 4-year period (111). Studies of children in Mexico City reveal that GSTM1 null children with asthma have decreased lung function associated with O₃ exposure compared to those who are GSTM1 sufficient. Interestingly, within this group, if one also screens for a second antioxidant SNP (the Pro187Ser SNP for NADPH quinone oxidoreductase 1 or NQO1), those with the Pro/Pro allele genotype for NQO1 have significantly reduced asthma risk ascribed to O₃ exposure, compared to GSTM1 null children with other NQO1 genotypes (109). Thus, while GSTM1 null state is associated with risk for O₃-induced asthma exacerbation, this risk may be mitigated by other protective genes. These studies collectively suggest that specific SNPs of GST genes modulate the response of asthmatics to a variety of oxidizing air pollutants.

Interventions for O₃ and LPS-induced disease

Mechanistic/pharmacologic interventions

Understanding potential mechanisms of the response to pollutants allows for rationale investigation of interventions which to mitigate the effect of O₃, endotoxin and other pollutants on lung disease. As noted above, endotoxin and O₃ exert effects on airway reflexes to cause nociceptive decrements in lung function, production of eicosanoids, and activation of inflammatory responses. Oxidative stress also impacts inflammatory processes and O₃ and endotoxin both exert an oxidant effect on the airway. O₃ is a direct oxidant, and both O₃ and endotoxin induce endogenous oxidants by inflammatory and epithelial cells. These observations point to a number of potential pharmacological

approaches to mitigate the effect of pollutants on the airway, including anti-inflammatory and antioxidant interventions. Although there have not been large scale clinical trials testing efficacy of specific agents for prophylaxis or treatment of pollutant-induced asthma exacerbation, there are intriguing studies which explore the potential of various treatments on responses to O₃ and endotoxin.

Analgesics

The nociceptive restriction in lung function observed after O₃ challenge is due to activation of C fibers. Among the mediators that can activate these nerves are PGE₂. It has been shown that spirometric decrements following O₃ exposure correlate with levels of PGE₂ in postchallenge BAL (34). Several studies in animals and volunteers have demonstrated that cyclooxygenase inhibitors, such as ibuprofen and indomethacin, prevent the occurrence of the nociceptive effect of O₃ on spirometry. However, non-steroidal anti-inflammatory agents have little effect on O₃-induced inflammation or increases in airway hyperreactivity (121–125). In addition to non-steroidal anti-inflammatory drugs, sufentanyl (a short acting narcotic) can mitigate the nociceptive response to O₃. Volunteers given sufentanyl shortly after O₃ challenge have a significant improvement in the nociceptive decrease in lung function (126). These studies demonstrate that analgesics may be useful in decreasing or reversing the immediate effect of O₃ on lung function.

Antioxidants

Given the role that oxidants play in the pathophysiology of asthma exacerbation, it seems likely that defects in antioxidant levels would be a risk factor for occurrence of acute asthma due to environmental factors. Nutritional deficiencies in antioxidants like vitamin E, ascorbate, and selenium have been linked to asthma exacerbation and severity (127–131). Additionally, persons with asthma have decreased levels of antioxidants in airway fluid. We have shown that ascorbate is decreased in airway fluids of asthmatics compared to normal volunteers (132), and others have shown decreases in serum ascorbate in asthmatics (133). Kelly *et al.* (134, 135) have shown that lung lining fluid ascorbic acid (vitamin C) and α -tocopherol (vitamin E) concentrations are low in patients with mild asthma even though blood levels are normal or increased. Taken together, these observations suggest that oxidant stress plays an important role in airway inflammation in asthma and that antioxidant supplementation should be beneficial in asthma.

These observations on nutritional antioxidants have led to the hypothesis that restoring or enhancing antioxidants in airway epithelial lining fluid may be important in blunting the effect of oxidizing pollutants, such as O₃ and particulates. Examples of antioxidants found in airway fluids include uric acid, ascorbic acid, tocopherols, and glutathione (136, 137). Exposure to O₃ has been reported to decrease airway urate levels and induce increases in glutathione or ascorbate, suggesting that urate may be absorbing oxidants and that glutathione and ascorbate are secreted into the airway to enhance antioxidant levels in the airway. However, these antioxidant responses do not correlate with prevention of O₃ induced pathophysiology (135, 137–140).

To test the idea that antioxidant supplementation may protect against the effect of O₃, Samet *et al.* (141) compared the effect of the effect of an ascorbate rich diet versus an ascorbate depleting diet on O₃ response in healthy volunteers. They reported a modest protection against O₃ induced decreases in lung function, but not in airway inflammation, suggesting that these antioxidants may be an possible prophylactic treatment for the impact of O₃ on lung function (141). The effect of vitamin E and C combination treatment on O₃-induced airway responsiveness was examined by Trenga *et al.* (130), who found a protective effect of this combination on airway reactivity in O₃-challenged asthmatics. Romieu *et al.* (127) examine the effect of combination ascorbate and α -tocopherol treatment in children with asthma. They observed that this treatment was associated with decreased occurrence of asthma exacerbation, as well as less pollutant-induced inflammatory changes in the nasal airway. It is noteworthy that those children whom seemed to benefit from antioxidants were those with the GSTM1 null genotype. This suggests that specific genetically defined populations may benefit from antioxidant interventions.

Inducers of the NRF2 transcription factor, which promotes increases in a large number of antioxidant enzymes, including GSTM1, have also been suggested as potential antioxidant treatments. While these have not yet been tested in human studies for efficacy, these are promising agents for prevention of pollutant-induced disease. α -Lipoic acid is a known inducer of NRF2. It has been shown that aging rats have impaired glutathione synthesis due to diminished gamma-glutamylcysteine ligase (GCL), the rate-controlling enzyme in GSH synthesis associated with decreased NRF2 activity and decreased constitutive phase II enzyme expression. This is attenuated by treatment with α -lipoic acid which up-regulates NRF2 activity in aging rats and restores GCL-associated glutathione synthesis (142) and loss of hepatocellular ascorbate (143). In cell cul-

ture studies, lipoic acid also protects against acrolein (a pro-oxidant agent abundant in tobacco smoke and diesel exhaust) related increases in intracellular oxidant levels and decreases in cell viability, GSH levels, antioxidant capacity, NRF2 expression, and phase II enzyme activity (144).

Sulforaphanes are also inducers of NRF2 derived from broccoli and other cruciferous vegetables. These have been shown to decrease oxidant and carcinogenic effects in a number of cell line studies (145, 146). It has been shown that sulforaphane prevents diesel-exhaust induced increases in both IgE production by B lymphocytes and inflammatory cytokine production by bronchial epithelial cells *in vitro* (147, 148). In humans, supplementation with broccoli-based preparations which are rich in sulforaphane markedly enhance NRF2 regulated antioxidant enzymes NQO1, GSTM1, GSTP1 and HO-1 in recovered nasal epithelial cells. In preclinical studies, sulforaphane has also been shown to inhibit endotoxin induced inflammatory responses. Studies of the effect of sulforaphane on rat microglia and murine microglia cell lines reveal that sulforaphane inhibits endotoxin-induced stimulated ERK1/2 and JNK phosphorylation with subsequent attenuation of NF- κ B and activator protein-1 (AP-1) activation (149). This treatment also inhibited endotoxin-induced expression of IL-1 β , IL-6, and TNF α . Similar effects are noted in endotoxin-stimulated peritoneal macrophages (150). Whether NRF2 activation is the only mechanisms by which sulforaphane acts is not clear (151). However, these results are consistent with the hypothesis that oxidative stress modulates inflammatory responses to endotoxin and O₃.

Anti-inflammatory agents

Given the pro-inflammatory actions of a number of pollutants, including NO₂, O₃, particulates and endotoxin, it is not surprising that agents with anti-inflammatory actions decrease the response to pollutants. Cromolyn sodium or nedocromil have both been shown to blunt immediate spirometric responses to endotoxin (152) in asthmatic volunteers. Corticosteroids have also been shown to blunt the effect of pollutants on airway inflammation. In non-asthmatic subjects, there are conflicting findings of the effect of corticosteroids on O₃-induced neutrophilic inflammation, though we observed that fluticasone inhibited O₃ induced neutrophilia (Fig. 3) in persons screened for O₃ responsiveness (44, 153, 154). Corticosteroids have been shown to decreased O₃-induced inflammation in allergic asthmatics (155).

Corticosteroids have also been examined for their impact on endotoxin-induced inflammation. Corticosteroid treatment

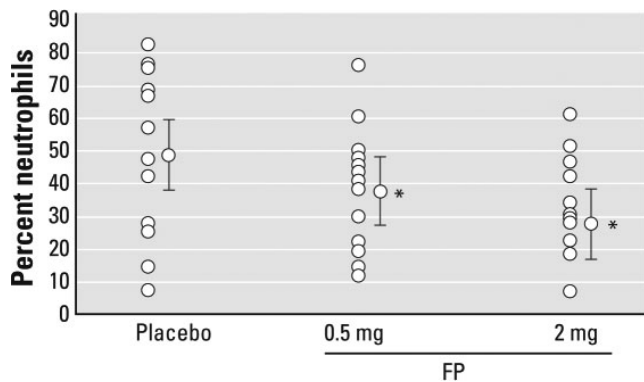


Fig. 3. The percent sputum neutrophils after O₃ exposure for each pretreatment dose of FP (0.5 or 2 mg) or placebo. **P* < 0.05 compared with placebo. From *Environ Health Perspect* 2008 June; **116**: 799–805. Published online 2008 February 28. doi: 10.1289/ehp.10981. Copyright: This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original DOI.

prior to inhalation challenge with grain-dust extract, an endotoxin rich preparation, has a mild protective effect against both inflammatory and bronchoconstrictive responses to such challenge in healthy subjects (156). Studies done by our group in mild allergic asthmatics demonstrate that pretreatment with inhaled fluticasone prior to challenge with 5 µg of nebulized endotoxin decreases both the expression of CD14 on airway monocytes and macrophages and the neutrophilic response to endotoxin in allergic asthmatics (63). As both endotoxin and O₃ induce airway neutrophilia, these evoked phenotypes are being employed to assess the effect of novel anti-inflammatory agents on airway disease.

Other novel anti-inflammatory agents are being developed which may be therapeutically efficacious and also confirm the role of specific inflammatory pathways which may mediate endotoxin and O₃ effects. A novel CXCR2 selective antagonist

was shown to inhibit O₃-induced airway inflammation in humans (157). Such an agent should inhibit endotoxin induced inflammation as well, given the effect of this agent on IL-8. A TLR4 antagonist has also been tested and is active in systemic endotoxin challenge studies (158–160).

Conclusion and future directions

Air pollution is a common cause of respiratory morbidity. O₃ and endotoxin (a component of indoor bioaerosols and ambient air particulate matter) are among the most common pollutants encountered by the US population. There is a remarkable similarity between the evoked phenotype response to O₃ and endotoxin, with both being characterized by influx of neutrophils, increases in cytokines like IL-1β, IL-6, and IL-8, increased expression of innate immune (CD11b, CD14, TLR4), antigen presenting (CD80, CD86, HLA-DR), and immunoglobulin receptor (CD16, FcεRI) proteins on airway macrophages and monocytes.

Both O₃ and endotoxin induce increased immediate and inflammatory response to inhaled allergen in allergic people. Together, these observations suggest that many common mechanisms mediate responses to both agents. There is evidence that TLR4 and inflammasomes modulate inflammatory response to O₃ and endotoxin, and that oxidative stress impacts inflammatory signaling processes. Mechanistic interventions should focus on anti-inflammatory and, antioxidant processes. It seems likely that as more damage associated molecular patterns which are produced in response to environmental injury are identified; more mechanisms by which O₃ and endotoxin exert their effects will be discovered. Promising new interventions include new anti-inflammatory molecules and anti-antioxidant moieties, including NRF2 inducers.

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