Ozone, NO, and NO$_2$: Oxidant Air Pollutants and More

Jerold A. Last,[1] Wei-Min Sun,[1] and Hanspeter Witschi[2]

[1]Department of Internal Medicine (Pulmonary Division), California Regional Primate Research Center, University of California, Davis, California; [2]Institute of Toxicology and Environmental Health, University of California, Davis, California

This article reviews the acute and chronic toxicity of the three oxidant air pollutants ozone, nitric oxide (NO), and nitrogen dioxide (NO$_2$). The toxicity of binary mixtures of NO$_2$ with other inhaled agents is also discussed. Newer studies are emphasized, especially those published in the last 5 years or still in press. Very recent data from our laboratory that suggest a new cellular mechanism of importance in lung injury in animals exposed to mixtures of ozone and NO$_2$ that may have general relevance with regard to the effects of oxidant air pollutants on the lung are also presented. — Environ Health Perspect 102(Suppl 10):179–184 (1994)

Key words: lung fibrosis, toxicologic interactions, lung disease

Nitric Oxide

Two or three years ago a discussion of the effects of NO with regard to lung injury would have been brief and simple. According to the U.S. EPA's Criteria Document for NO$_x$ (1), "the toxicological data base for NO is not extensive except for its interaction with blood [and] ...high exposure levels are needed [to see] significant changes." NO has been estimated to be about 30 times less toxic than NO$_2$, based upon pulmonary responses to acute exposures (1). Since most of the (few) chronic and subchronic experiments testing the effects of NO on experimental animals used atmospheres also containing low levels of NO$_2$ (as an impurity arising from the reaction $2NO + O_2 \rightarrow NO_2$), it is difficult to interpret these early studies (2,3). Air quality standards for NO include a time-weighted average exposure level of 25 ppm or less, with a concentration of 100 ppm not to be exceeded for any 15-min interval. These values should be compared with the estimated 400 to 1000 ppm of

NO per puff of cigarette smoke delivered to the lungs.

However, since this document appeared, NO has become one of the most intensively studied molecules in recent biology, causing it to be honored as *Science's* "Molecule of the Year" in 1992. What happened? NO, which arises in the body by the action of nitric oxide synthetase on its substrate L-arginine, is a biologically active molecule. It is apparently identical to the endothelial cell-derived relaxing factor (EDRF). It also binds avidly to heme, as in its well-known reaction with hemoglobin to form methemoglobin, with an affinity for hemoglobin about 1500 times greater than that of carbon monoxide (CO). The same avidity for heme allows it to bind to the heme moiety of cytosolic guanylate cyclase, stimulating the formation of cyclic GMP. Via this and other pathways, NO exerts profound physiologic effects on lung, liver, pancreas, uterus, peripheral nerves, brain, the immune system, and especially blood vessels. NO can produce localized vasodilation, with decreased blood pressure, as it diffuses from arterial endothelial cells that synthesize it to vascular smooth muscle cells, which respond by producing additional cGMP. Because of its high rate of reaction with hemoglobin, NO does not diffuse far in blood and the effects of NO on blood vessels only occur locally over small regions. NO is currently being evaluated clinically for a possible role in the treatment of pulmonary hypertension in several syndromes, including adult respiratory distress syndrome (ARDS) and persistent pulmonary hypertension of the newborn (4). It is thought that the vasodilatory response of both the bronchial and pul-

monary vasculature to cigarette smoke is directly attributable to its NO content (5).

Lung cells other than endothelial, such as alveolar macrophages and fibroblasts, also can produce NO, and may thus act as effector cells on smooth muscle and other putative target cell populations. Interestingly, synthesis of NO by rat lung fibroblasts is stimulated by gamma interferon, and this stimulation is potentiated by lipopolysaccharide or by interleukin 1β (6).

Ozone

Several recent reviews of the effects of ozone on the lung have appeared (7–9), and the reader is referred to these sources for citations of original research in the older literature. This review focuses primarily on more recent studies that have opened new avenues for examination of the effects of ozone on the lung.

The National Ambient Air Quality Standard (NAAQS) for ozone is a peak hourly concentration of 0.12 ppm (235 mg/m^3), not to be exceeded twice in one year. The State of California standard is 0.09 ppm (180 mg/m^3). Both standards are routinely exceeded about half of the days each year in California's south coast (Los Angeles) air basin, with Stage 1 smog alerts (0.20 ppm) common in the summer months. Mexico City routinely exceeds these levels essentially every day of the year (estimated 360 days last year). For further details of ozone occurrence and areas in which standards are exceeded in the United States, the reader is referred to the U.S. EPA Air Quality Criteria Document for Ozone, published in updated form in 1994.

Acute exposure of laboratory animals to high concentrations of ozone (2 ppm and above) causes severe, often fatal, pulmonary edema (10). Exposure to concentrations of ozone between about 0.2 ppm (or less) and 1.0 ppm causes effects on the nasal epithelia (11) and on the lung, including mild edema, inflammation, damage to airway epithelial cells, and respiratory bronchiolitis (12–14) at the histologic level. Toxicologic responses of rats to ozone exposure in this concentration range include pulmonary edema, evaluated as increased lung wet weight or as increased protein content of lung lavage fluid (15), and increased synthesis rate of lung collagen (16,17).

Chronic exposure of laboratory animals to concentrations of ozone between 0.2 ppm (or less) and 1.0 ppm causes sustained respiratory bronchiolitis, extension of terminal bronchioles into the alveolar zone to form respiratory bronchioles in rats (a phenomenon known as bronchiolization), and centriacinar fibrosis (18–21). Based upon recent studies sponsored by the Health Effects Institute (HEI) (some yet to be published) of Fischer 344 rats exposed for 20 months to 0.12 ppm of ozone, bronchiolization may occur in lungs of animals exposed to concentrations of ozone at or near the NAAQS (21). Biochemical studies also have shown fibrosis in rats and monkeys chronically exposed to ozone (22–25). Fischer 344 rats exposed for 20 months (6 hr/day, 5 days/week) to ozone at concentrations of 0.12, 0.5, or 1 ppm show changes in lung structure. In the rats exposed to 0.5 and 1 ppm ozone, histologically demonstrable pulmonary fibrosis was also observed (26). We have since repeated this HEI protocol to perform an exposure of rats for 90 days to either 0.12 or 1 ppm ozone, in an attempt to determine whether the lesions seen after 20 months were apparent after 90 days. Were

that the case, there is an extensive database on responses of rats to ozone exposure over a 90-day period with which these results could be directly compared. In addition, some indication of the time course of development of the lesions could be inferred, since the original HEI protocol only allowed for sampling at a single time point, 20 months after initiation of exposure.

Biochemical analysis of lungs from the exposed rats showed no significant changes in hydroxyproline, a marker for collagen content (Figure 1). Nor was any change found in the lung content of hydroxypyridinium, a trifunctional collagen cross-link known to be increased in lung fibrosis (Table 1). While histologic and morphometric analysis of these lungs are still in progress, it is already clear that the rats exposed to 1 ppm ozone show changes in their centriacinar region consistent with bronchiolization, perhaps of comparable severity to the rats exposed for 20 months. These observations suggest the relative insensitivity of total lung hydroxyproline assays for measuring chronic effects of ozone exposure on rat lungs, and raise fascinating questions about continued progression of lung injury with continued exposure to ozone versus adaptation of the lungs after chronic exposure.

The rate of epithelial cell turnover is increased in conducting and peripheral airways of animals exposed to ozone (27), suggesting that ozone might act as a promoter of carcinogenesis in this target cell population. Small increases in lung tumor incidence have been reported in Strain A mice (but not in the Swiss Webster strain) exposed to ozone (28,29). Thus, based on these theoretical and experimental arguments, ozone was nominated for testing as a carcinogen or a co-carcinogen in a recently completed NIEHS National Toxicology Program-sponsored bioassay in rats and mice. The results of these experiments were released in November 1993 (29) and should allow an estimate of the extent of cancer risk (if any) associated with inhalation of ozone.

Nitrogen Dioxide

The National Ambient Air Quality Standard for NO_2 is an arithmetic mean annual average not to exceed 0.053 ppm (100 mg/m^3). There is a great deal of debate about the necessity for a short-term federal standard to regulate peak concentrations over an interval of one or a few hours. California has a peak hourly standard of 0.25 ppm (470 mg/m^3), suggesting an appropriate short-term standard were the EPA to take action in this area. Neither the national nor the California standards as they currently exist are routinely exceeded, i.e., most of the outdoor air in the United States is in conformity with these standards.

Acute exposure of humans to NO_2 at concentrations above about 150 ppm (282 mg/m^3) causes death, either rapidly due to pulmonary edema or after a few weeks due to bronchiolitis obliterans with severe fibrosis. In animal experiments, exposure of rats to about 25 ppm (47 mg/m^3) for several days is lethal to more than half of the animals. Exposure of rats to concentrations between about 4 and 20 ppm of NO_2 for several days causes mild edema, increased protein content of lung lavage fluid, inflammation, damage to airway epithelial cells, and bronchiolitis. By these criteria, there is a NOEL for NO_2 in animal experiments at about 2 ppm (30,31).

The effects of chronic exposure of experimental animals to NO_2 have been relatively less extensively studied than have been effects of ozone. However, there is extensive older literature suggesting that chronic exposure to high concentrations of NO_2 results in emphysema (31–33). More recent work on the effects of chronic exposure of specific pathogen-free rats to carefully controlled atmospheres has called this conclusion into question, and has suggested that, like ozone, chronic exposure of rats to high concentrations of NO_2 causes centriacinar fibrosis (34,35).

NO_2 is a far less toxic compound than ozone in animal experiments (31). On a molar ratio basis, ozone is about 18 times more damaging to the lungs of rats than is NO_2, as evaluated histologically (36) or by more quantitative biochemical measurements (16). It is not immediately obvious why this disparity in potency is observed as both ozone and NO_2 are highly reactive oxidants that share the property of probably being far too reactive to penetrate the
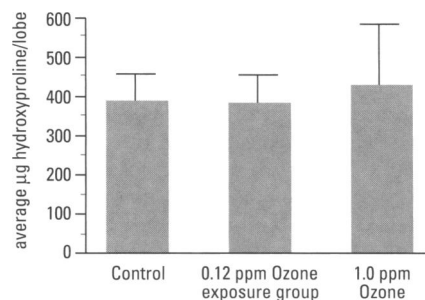

Figure 1. Hydroxyproline content of right medial lung lobe from rats exposed for 90 days to filtered air, to 0.12 ppm of ozone, or to 1 ppm of ozone. See Table 1 for details of exposure protocol. Data are presented in this and all subsequent figures as mean values ± 1 SD.

Table 1. Collagen content of lung lobes from rats exposed to ozone for 90 days.[a]

Ozone concentration, ppm (nominal)	Hydroxypyridinium content of right medial lobe, nmole (n)
0	2.4 ± 0.8 (11)
0.12	2.1 ± 0.9 (10)
1	2.5 ± 0.8 (10)

[a]Fischer 344 rats were exposed to the indicated (nominal) concentrations of ozone for 6 hr/day (9:00–15:00), 5 days/week. Actual ozone concentrations in the chambers were 0.12 ± 0.02 ppm and 1.00 ± 0.03 ppm. Data are presented as mean values ± 1 SD with n, the number of animals studied, in parentheses.

lung fluid lining layer intact (*37*). Both gases are insoluble enough to penetrate to the deep lung, and both seem to elicit their major effects in the centriacinar region (*38*). One important difference is that ozone dissolves in water as ozone, while NO_2 probably dissolves in water as its reaction products in a complex mechanism of reactive uptake (*39,40*). In addition, unlike ozone, some NO_2 is known to reach the blood, where it reacts with hemoglobin to form methemoglobin. In part because of its relative insolubility that confers upon it the ability to reach the alveoli, NO_2 has been suggested, but not shown, to cause emphysema after chronic exposure. Based upon observations made after accidental exposure of humans to lethally high concentrations, NO_2 is known to cause bronchiolitis obliterans and bronchiolar fibrosis (*31*).

Both NO_2 and ozone exposure cause oxidative stress to the lung, especially in the small airways and centriacinar regions where peak doses are received (*38*). Such oxidative stress can arise directly from the high reactivity of these two oxidant gases and their secondary reaction products with lung-lining fluids or cell membranes (*37,41*). Alternatively, additional oxidative stress can arise indirectly from superoxide ion, hydrogen peroxide, and other oxidants produced by phagocytosis of cell debris by inflammatory cells, especially alveolar macrophages and polymorphonuclear leukocytes. As discussed by several participants in this symposium, there are profound effects on the cells of the lung arising from attack by such oxidants. This is a complex area of research and details of specific cellular targets of oxidative stress and the relevant mechanisms of action are currently under active study in a large number of laboratories (*42*).

Existing air quality standards for ozone and NO_2 are currently justified by data from controlled human exposures showing responses to acute (hours) challenges (in normal or susceptible subjects) at or near the level of the standard. These values are also consistent with NOELs found in animal experiments using many conventional toxicologic, biochemical, and histologic responses to acute exposure regimens (hours or days). This is perhaps not surprising in that the pulmonary function tests used to evaluate responses of humans to controlled exposures tend to reflect cellular inflammatory responses in the lung (*43*). It is widely believed that the current standards are protective of human health based upon acute effects upon human volunteers receiving controlled exposures,

although many people have questioned whether the current standards for ozone allow for any margin of safety, much less the adequate margin of safety that the Clean Air Act requires (*7*). On the other hand, little or no explicit consideration of potential chronic effects on lung structure of exposure to ozone or NO_2 has gone into the setting of air quality standards, in part because of the paucity of data available to guide such a standard-setting exercise. As the potential for ozone or NO_2 to cause pulmonary fibrosis after chronic exposure has become more widely appreciated, concern about long-term effects of chronic low level exposure to ozone has increased. In this context, the apparent greater inherent susceptibility of nonhuman primates to ozone as compared with rats (*44*) also raises concerns, as chronic exposure (20 months) of rats to 0.5 ppm of ozone (or less) causes pulmonary fibrosis (*26*).

Mixtures of Ozone and NO_2

About 40 years ago Diggle and Gage (*45,46*) suggested that mixtures of ozone and NO_2 might react to form nitrogen pentoxide, N_2O_5, that they suggested might be more toxic than either ozone or NO_2. We have recently reinvestigated the possibility of interactions between ozone and NO_2 in a series of studies encompassing responses of rats to both acute and chronic exposures. In addition to confirming the suggestions of Diggle and Gage, we have observed several additional responses of the exposed rats that are of interest.

Acute exposure of rats to mixtures of ozone and NO_2 elicits a synergistic response to the mixtures at high dose rates (*47,48*). This response was greater than the sum of the responses to either oxidant gas alone, and represented true synergy (*49*). The exact exposure protocols eliciting this synergistic interaction varied by assay used, with differing sensitivities observed when the end point measured was related to edema or to cellular inflammatory response. Many other investigators have exposed laboratory rodents to mixtures of ozone and NO_2 (*36,50,51*); our experiments differed in that we used very high ratios of NO_2 to ozone (18:1) to elicit equal toxic responses from each of the two oxidant gases (*16*).

Subchronic exposure of rats to mixtures of ozone and NO_2 results in a syndrome unlike that elicited by either agent alone. Relatively low concentrations of the two gases together (e.g., 0.4 ppm ozone + 7.2 ppm NO_2, 12 hr/day, exposure intervals of about 7 to 10 weeks), cause substantial numbers of deaths in rats (*35*). Lung colla-

gen content is significantly elevated in rats exposed for 78 to 90 days to either 0.8 ppm ozone and 14.4 ppm NO_2 (6 hr/day), or to 0.2 ppm ozone and 3.6 ppm NO_2 (24 hr/day; the same C × T product in both regimens). The animals exposed to 0.8 ppm ozone and 14.4 ppm NO_2 had severe pulmonary fibrosis as evaluated histologically, while the animals exposed to 0.2 ppm of ozone and 3.6 ppm NO_2 did not (*34*). These lesions are apparently irreversible, as other groups of rats exposed to the same C × T product (0.4 ppm ozone and 7.2 ppm NO_2, 12 hr/day; or 0.6 ppm ozone and 10.8 ppm NO_2, 8 hr/day) for 45 days, then allowed to recover in filtered air for 45 or 60 days, showed continued accumulation of collagen in their lungs during the postexposure recovery period. (Last et al., manuscript in preparation.)

These observations give rise to a new animal model of progressive pulmonary fibrosis and to the fascinating question of what is occurring in the lungs of these rats over the 6- to 8-week interval before the damage progresses far enough to kill the animal. Coupled with these questions is the observation that total dose of ozone plus NO_2 (as C×T product) is not the critical determinant of lung injury in this model; rather, the combination of repetitive exposure periods and recovery periods seems to be essential for lung damage. To test this hypothesis we exposed five groups of rats for 3 days to different C×T products of ozone and NO_2 combined (in multiples of 4.8 ppm/hr and 86.4 ppm/hr, respectively) at four different total doses. This is a different approach from our earlier experiments (*47*), where dose rate was varied by delivering a constant C×T product for different intervals of time each day. Thus, we exposed rats for 3 days to nominal concentrations of 0.8 ppm ozone plus 14.4 ppm NO_2, and varied the duration of exposure as follows: group 1, 0 hr/day (controls); group 2, 6 hr/day; group 3, 12 hr/day; group 4, 18 hr/day; group 5, 24 hr/day. Thus, with respect to C×T product, groups 4 through 1 received 4*x*, 3*x*, 2*x*, and 1*x*, where *x* = 4.8 ppm/hr + 86.4 ppm/hr of ozone and NO_2, respectively. We would, therefore, predict that if total dose alone determines lung damage, we would find damage in the ratio of 4:3:2:1 in the four groups of exposed animals. Interestingly, that is not what we observe. Rather, as shown in Figure 2, there seems to be a peak of lung damage in group 3, as evaluated by assay of the protein content of lung lavage fluid, despite the fact that group 3 received only half the total dose (C×T product) as
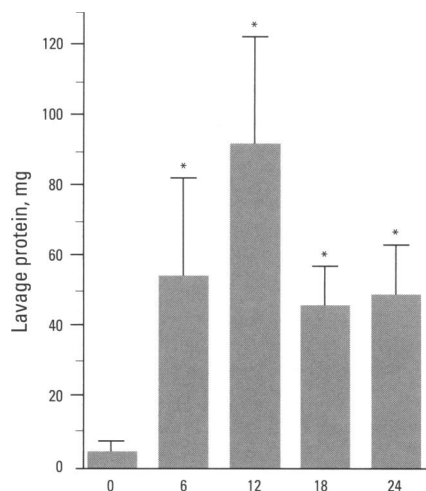
Figure 2. Protein content of lung lavage fluid from rats exposed for 3 days to a mixture of (nominal concentrations) 0.8 ppm ozone + 14.4 ppm NO_2 for the indicated duration of exposure each day. Asterisk indicates a significant increase above control values (Fisher PLSD test). Actual concentrations of pollutants were as follows: group 2 (6 hr/day) 0.84 ± 0.03, 14.0 ± 0.7 ppm; group 3 (12 hr/day) 0.79 ± 0.04, 14.0 ± 1.1 ppm; group 4 (18 hr/day) 0.86 ± 0.04, 14.4 ± 0.5 ppm; group 5 (24 hr/day) 0.85 ± 0.03, 13.9 ± 0.7 ppm.
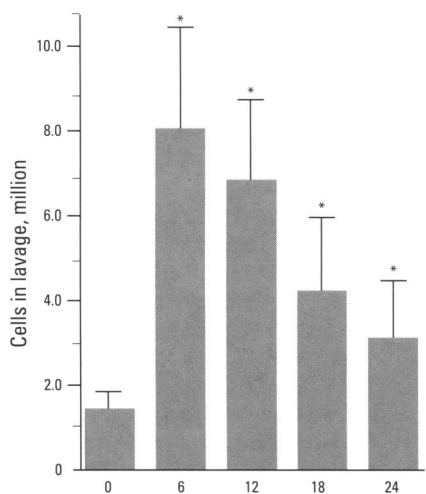

Figure 3. Total lavagable cells from the same rats exposed for 3 days to the mixture of ozone and NO_2 detailed in Figure 2.

Figure 4. Differential analysis of the cell content in the lavage pellet from the rats described in Figures 2 and 3.

did group 5 and 3/4 of the total dose as did group 4. The protein content of lavage fluid in group 3 is significantly higher than that of groups 2, 4, and 5 (Fisher PLSD test). All of the exposed rats weighed significantly less than the controls.

We have also examined the cell content of the pellets obtained from the lung lavage fluid of the same animals (Figures 3,4). There were significantly more cells in each of the exposure groups than in the controls,
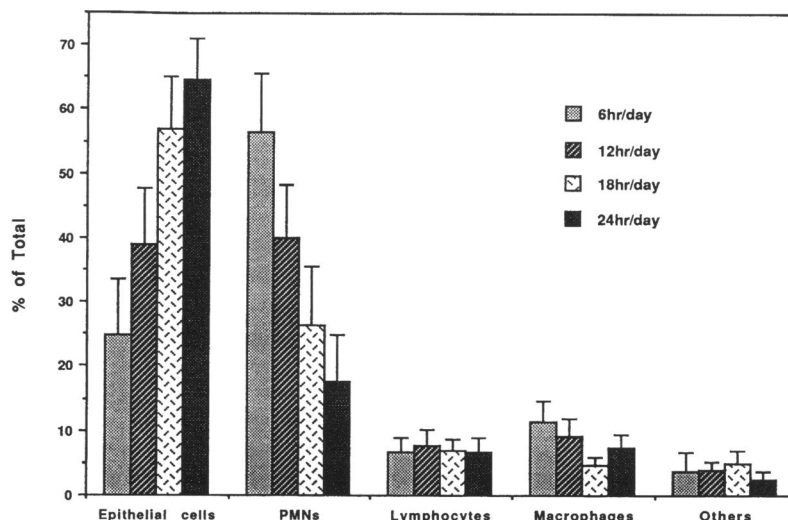
and the increased cellularity was inversely proportional to the duration of exposure. Thus, groups 2 through 5 had about 475, 380, 200, and 110% increases in cell number above the control value, respectively. The total cell numbers were not significantly different when groups 2 and 3 (6 and 12 hr/day) were compared, but both of these groups had significantly greater numbers of cells in their lavage fluid than did the groups 4 and 5 (18 and 24 hr/day) animals. The increased cell number in these lavages is due to increases in essentially two populations: epithelial cells arising from damage to the airway epithelium, and polymorphonuclear leukocytes arising from the inflammatory response of the lung to injury (Figure 4). Interestingly, neutrophils follow the same duration-response curve as does the total cellularity of the lavage fluid: response is inversely proportional to the duration of exposure, with groups 2 and 3 not significantly different from each other but both groups significantly greater than groups 4 and 5 (Figure 4). Conversely, the epithelial cell content of the lavage fluid appears to be directly proportional to the duration of exposure (Figure 4), with the higher total C × T product producing more direct damage to the airway epithelium.

These observations suggest that, as we would assume intuitively, the longer the duration of exposure to the mixture of ozone and NO_2, the greater the effective dose delivered to the airway epithelium and the greater the amount of direct damage to the epithelium as evaluated by sloughing of epithelial cells into the lung-lining layer. But then something unexpected happens. Our measurements for whole lung damage,

protein content of lavage fluid supernate, and total cell content of lavage fluid pellet (which measure lung edema and inflammation, respectively) show a very different pattern of response. The animals exposed intermittently for 12 hr/day (group 3) show a significantly greater response than do those that were exposed to 50 or 100% more ozone + NO_2 (groups 4 and 5, respectively). Thus, our attention has begun to focus on the importance of the recovery period, rather than the exposure duration, in eliciting lung injury with these two oxidant gases. Our current working hypothesis is that repeated episodes of exposure and recovery (e.g., the regimen received by group 3 in these experiments) are more damaging to the lung than is continuous exposure (i.e., group 5) to the mixture of ozone and NO_2. A similar phenomenon may also occur in lungs of rats exposed to ozone alone (23,24).

What might be the cause of repeated intermittent exposures to ozone or to ozone and NO_2 being more damaging to the lung than continuous exposure to higher total doses of the pollutants? We speculate that exhaustion of some essential antioxidant defense (52) or of some essential precursor cell pool plays a critical role in the lung's increased susceptibility to intermittent exposure regimens with alternating cycles of damage and repair. This is a current area of active investigation in our laboratories.

An interesting curiosity that probably has little real world significance is a synergistic interaction between NO_2 and NaCl aerosol we described several years ago (30). This interaction almost certainly arises

from the chemical reaction between NO_2 and NaCl to form nitrosyl chloride, NOCl, the putative toxic agent. This interaction, while probably requiring higher concentrations of both reactants than occur in polluted atmospheres, however highlights an important mechanism of synergis-

tic interaction when reactive oxidants such as NO_2 are involved, the chemical reaction of components of a mixture to form a new, more toxic, chemical species. We have already discussed the interaction of ozone and NO_2 as another example of this mechanism of synergy. Thus, questions of ade-

quate margins of safety for current air quality standards are also made much more complicated by the issue of whether oxidant gases may be more toxic as part of a mixture than when delivered alone, as is normally done in controlled exposures to human volunteers or to experimental animals.

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