Detection of Aldehydes in Bronchoalveolar Lavage of Rats Exposed to Ozone

WILLIAM A. PRYOR, ELMER BERMUDEZ, RAFAEL CUETO, AND GIUSEPPE L. SQUADRITO

Biodynamics Institute, 711 Choppin Hall, Louisiana State University, Baton Rouge, Louisiana 70803-1800

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We report the detection of hexanal, heptanal, and nonanal in the bronchoalveolar lavage (BAL) of rats exposed to 0.5 to 10 ppm ozone with or without simultaneous 5% CO₂. Three of these aldehydes primarily result from the Criegee ozonation of specific mono- or polyunsaturated fatty acids that are present in significant amounts in the rat lung: e.g., palmitoleic acid gives heptanal, oleic gives nonanal, and linoleic and arachidonic can give hexanal. Hexanal also is produced in the ozone-initiated autoxidation of any n-6 polyunsaturated fatty acid, and thus is a measure of generalized oxidative stress. (Mono- and polyunsaturated fatty acids do not undergo appreciable autoxidation.) This detection and quantitation of aldehydes directly demonstrates for the first time that unsaturated fatty acids undergo Criegee ozonation in the lung when ozone is inhaled. Exposure to ozone alone produced smaller apparent yields of the three aldehydes than did exposure to ozone plus 5% CO₂. Hexanal, heptanal, and nonanal can be detected in BAL of rats 5 hr after the end of the ozone exposure, but after more than 5 hr only hexanal can be found, probably from ozone-induced autoxidation of n-6 PUFA that continues after ozone exposure. The measured amounts of aldehydes are low, and that, coupled with inherent biovariability, suggests that aldehydes may not be useful as quantitative dosimeters. However, they can be useful biomarkers, since some of these aldehydes (e.g., nonanal) are produced in ozone-specific pathways and aldehydes are the most easily detected among the lipid ozonation products (LOP). Furthermore, our identification of these aldehydes by BAL, coupled with our recognition that ozone itself cannot penetrate far enough into the lung to cause many of the effects associated with the inhalation of ozone, suggests that these aldehydes, as well as other types of LOP (such as hydroxyhydroperoxides and Criegee ozonides), may act as signal transduction molecules, activating lipases and causing the release of inflammatory molecules by a variety of pathways not yet entirely elucidated.

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1 To whom correspondence should be addressed. Fax: (504) 388-4936. E-mail: WPryor@unix.lsncc.LSU.edu.

2 Current address: Department of Health and Safety, Arena B-83, Indiana State University, Terre Haute, Indiana 47809.

Ozone, which occurs at ppm levels in photochemical smog, is the most powerful oxidant to which humans are routinely exposed and presents a major health problem (Lippman, 1989; Pryor, 1993). Animals exposed to ozone are known to undergo extensive damage to cell membrane lipids in the centriacinar region deep in the bronchopulmonary tree (U.S. Environmental Protection Agency, 1986; Cripo et al., 1992).

Ozone itself is too reactive to penetrate far into the pulmonary air/tissue boundary before reacting (Pryor, 1992). Therefore many of the pulmonary and all of the nonpulmonary effects of ozone must arise from the production of initial ozonation products as ozone enters the lung/air interface and the subsequent reactions of these initial ozonation products. The initial ozonation products are known to include molecules formed from the ozonation of lipids (Goldstein and Balchum, 1967; Goldstein et al., 1969; Balchum et al., 1971; Mudd and Freeman, 1977; Pryor et al., 1976; Leikau et al., 1993). These primary reaction products then relay the effects of ozone to deeper tissue strata (Pryor, 1993; Goldstein and Balchum, 1967; Pryor et al., 1995b; Leikau et al., 1995; Wright et al., 1994).

As we first suggested in 1991, it is likely that the species that relay the effects of ozone include lipid ozonation products (LOP). First, unsaturated fatty acids in lung lipids have long been considered to be a primary target for ozone (Pryor and Church, 1991; Pryor et al., 1991a; Goldstein and Balchum, 1967; Goldstein et al., 1969; Balchum et al., 1971; Mudd and Freeman, 1977). The epithelial cell-lining fluids (ELF) in the lung, including mucous and surfactant, contain from 20 to 40% unsaturated fatty acid-containing lipids (Harwood et al., 1975; Shelley et al., 1984; King, 1974; Sahu et al., 1976). Second, lipids, in contrast to proteins or nucleic acids, give small, discrete, diffusible products on ozonation (Bailey, 1978; Uppu and Pryor, 1994). Third, we have detected significant increases in the amounts of aliphatic alde-
hydes in the bronchoalveolar lavage of rats following ozone exposure (Cueto et al., 1992, 1994). Finally, oxidized lipids are known to act as messengers of a variety of cellular signals, including the inflammatory response (Pryor et al., 1995b; Wright et al., 1994b; Leikauf et al., 1993, 1995; Stremler et al., 1989; Zimmerman et al., 1995).

Among the types of LOP that are produced, we have suggested that aldehydes, hydroxyhydroperoxides, and Criegee ozonides are likely mediators of ozone toxicity (Pryor and Church, 1991; Pryor et al., 1991a,b). Aldehydes are of special interest, since the ozonation of unsaturated fatty acids in models of the ELF predicts that aldehydes are formed in yields of about 90% (along with hydrogen peroxide and some hydroxyhydroperoxides) and Criegee ozonides in about 10% yields (Pryor and Church, 1991; Pryor et al., 1991a; Pryor, 1994; Squadrito et al., 1992b). Aldehydes are also the most easily detected of the LOP formed at low levels in biologically relevant in vivo exposures. Aldehydes also are formed in the autoxidation of polyunsaturated fatty acids (PUFA), but monounsaturated fatty acids do not undergo rapid autoxidation (Pryor et al., 1976; Cosgrove et al., 1987). Thus, the aldehydes produced from the ozonation of monounsaturated fatty acids in ELF and pulmonary lipids would appear to be promising and specific biomarkers of ozone exposure (Pryor et al., 1995b).

The structures of the products produced when an unsaturated compound undergoes ozonation can be predicted from the Criegee ozonation mechanism (Bailey, 1978; Pryor et al., 1995b). When an unsaturated fatty acid (UFA) undergoes ozonation, one end of the double bond becomes an aldehyde and the other an unstable carbonyl oxide that rapidly hydrolyses to give a hydroxyhydroperoxide; Scheme 1 shows Criegee ozonation of an olefin and the aldehyde, hydroxyhydroperoxide, and ozonide products that are formed. As is also shown in the scheme, the hydroxyhydroperoxide is in equilibrium with another mole of aldehyde and a mole of hydrogen peroxide.

When an unsaturated fatty acid attached to a lipid backbone undergoes ozonation, either the aldehydic function remains attached to the lipid and a hydroxyhydroperoxide liberated or vice versa (see Scheme 2). Thus, the species that are released include small aldehydes, aldehydes attached to the lipid backbone, small hydroxyhydroperoxides, or hydroxyhydroperoxides attached to the lipid (Pryor and Church, 1991; Pryor et al., 1991a,b; Pryor and Wu, 1992; Squadrito et al., 1992b). In addition, a small yield of the Criegee ozonide is produced (see Scheme 2).

If we consider the structures of just the free (i.e., not attached to the lipid backbone) aldehydes that are produced, only a small number of aldehydes can be formed from the fatty acids present in the ELF and lung tissue. Among the UFA that are the most prevalent in the lungs of both rats and humans, ozonation of linoleic acid yields hexanal and 3-nonenal; ozonation of arachidonic gives hexanal, 3-nonenal, 3,6-dodecadienal, and 3,6,9-pentadecatrienal; ozonation of palmitoleic gives heptanal; and ozonation of oleic gives nonanal. In addition, cholesterol produces secoaldehyde (Pryor et al., 1992).

For an aldehyde to be produced in appreciable yield, the precursor fatty acid must be present in the ELF in significant amounts. The percentages of various unsaturated fatty acids in rat ELF are shown in Table 1 and the aldehyde products that are predicted to be formed in the Criegee ozonation of those fatty acids are listed in Table 2. The data in Table 2 predict that the relative yields of saturated aldehydes in the lavage of rats exposed to ozone should be hexanal > nonanal > heptanal; as will be seen, our results support this prediction. A number of unsaturated aldehydes also would be expected to be formed on ozonation of ELF lipids, and these might be specific biomarkers for ozone since they are formed with the double bond and the carbonyl group not in conjugation, whereas the ozone-initiated autoxidation of PUFA would produce alkenals that have a double bond conjugated with the carbonyl function. However, we elected not to attempt to detect these nonconjugated alkenals since they are thermodynamically unstable relative to the conjugated isomers and the conjugated isomers themselves are reactive (for example, by Michael addition of thiols).

In this paper, we show that when rats are exposed to ozone concentrations as low as 0.5 ppm for 30–60 min, the amounts of aldehydes in the bronchoalveolar lavage (BAL) of the rats increase. In addition, data are presented on the rate of disappearance of these aldehydes from the ELF, as detected by the analysis of lavage, at times up to 24 hr after the end of the ozone exposures. When CO₂ is included in the breathing air, it increases the rate of ventilation and therefore increases the uptake and deposition of a toxicant that is coadministered (Tepper et al., 1988). Therefore, we used 5% carbon dioxide in the breathing air, and we find, as expected, that the apparent yields of aldehydes are higher when rats are exposed to ozone plus CO₂ than those with ozone without CO₂.
SCHEME 2. Ozonation of an unsaturated fatty acid-containing lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), showing the products that remain attached to the lipid backbone and those that are liberated. The abbreviation system used is: HHP-C9 and Ald-C9 are the 9-carbon hydroxyhydroperoxide and aldehyde, respectively, and PC-Ald and PC-HHP are the lipids with an aldehydic or hydroperoxide function at the terminus of the shortened fatty acid chain at the 2 position. The POPC-Oz is the Criegee ozonide of the original POPC lipid. The double bonds that do not undergo ozonation retain their cis configuration.

MATERIALS AND METHODS

Materials

The chemicals utilized in this study were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Plastic syringes were obtained from Baxter Co. and rat food was from Harlan Teklad (Madison, WI).

Methods

Animal exposures. Ninety-day-old male Sprague-Dawley specific-pathogen-free rats weighing 300—330 g (Harlan Sprague-Dawley, Houston, TX) were used. Rats were acclimatized in standard cages with access to food (Harlan Teklad) and water for at least 5 days prior to exposure. Twelve-hour day/night lighting intervals were maintained. Rats were randomized into control and experimental groups. The animals were weighed and then transferred to stainless-steel open-mesh cages (one per cage) and placed in the exposure chamber as described by Hinners et al. (1968). The rats were exposed to filtered air, filtered air plus 5% CO₂, ozone, or ozone plus 5% CO₂. Ozone concentrations of 0.5, 1.2, 2.5, 5.0, and 10.0 ppm were used and exposure times of 30, 60, 90, 120, and 240 min. Animals had access to water during the exposure but not food.

The chamber was a 0.25-m³ whole-body exposure chamber (Air Dynamics, Inc., Baton Rouge, LA). The air flow rate was adjusted to give 12 chamber volume changes per hour. Ozone was generated by passing compressed air (1.0 liter/min) through a Sander Ozonizer (Model 200, Sander Aquarientechnik, AM Osterberg, Germany) and then diluted with filtered room air to the desired concentration. The concentration of ozone in the exposure chamber was monitored continuously via a probe in the geometrical center of the chamber using an ozone analyzer (Dasibi Model 1008-AH, Dasibi Environmental Corp., Glendale, CA) connected to a strip chart recorder. The ozone analyzer was calibrated using a calibration kit (Enmet Analytical, Ann Arbor, MI). The chamber was tested for the homogeneity of the distribution of ozone concentrations. Carbon dioxide was produced from a pressurized cylinder of CO₂ and diluted to the desired concentration with filtered air; the flow of CO₂ into the chamber was 0.5 liter/min. The CO₂ concentration was continuously monitored using a Beckman medical gas analyzer Model LB-2 (Beckman Instruments, Fullerton, CA) connected to a strip chart recorder. The CO₂ analyzer was calibrated using a standard of 1.2% CO₂ in nitrogen (Aldrich, Milwaukee, WI).

All the procedures used in this study were reviewed and approved by the LSU Institutional Animal Care and Use Committee.

<table>
<thead>
<tr>
<th>Unsaturated fatty acid*</th>
<th>Percentage of total FA,s</th>
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<tbody>
<tr>
<td>16:1 (n-7)</td>
<td>9.2</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>10.8</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>10.0</td>
</tr>
<tr>
<td>20:3 (n-9)</td>
<td>0.3</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>7.6</td>
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</tbody>
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* Fatty acids are named by giving the total number of carbon atoms; the number of double bonds (the carbon atom from the end on which the terminal double bond ends).
Ozonation-Derived Aldehydes in Lavage

Aldehyde Production and the Effect of CO₂

We first tested the efficacy of using the combination of 5% CO₂ with ozone in order to increase the tidal volume of the rats and therefore increase the apparent yields of aldehydes in the BAL. Figures 1A and 1B show the amounts of hexanal, heptanal, and nonanal detected in the BAL of rats exposed to 2.5 ppm ozone and 2.5 ppm ozone plus 5% carbon dioxide, respectively. Exposure to ozone alone gave smaller amounts of hexanal, heptanal, and nonanal than those found when the rats were exposed to ozone plus 5% CO₂; this is the effect that was expected and can be attributed to the deeper and more frequent breathing of rats on 5% CO₂. It is known that concurrent exposure to carbon dioxide with a toxicant gas such as ozone increases the uptake and pulmonary deposition of the toxicant gas (Tepper et al., 1988). This use of carbon dioxide is analogous to human pollutant studies that use exercise to augment ventilation.

These aldehydes result from the Criegee ozonation of unsaturated fatty acids, as indicated in Schemes 1 and 2. The most prevalent unsaturated fatty acids in the rat lung are oleic, palmitoleic, linoleic, and arachidonic, as shown in Table 1. Heptanal arises from the ozonation of palmitoleic acid and nonanal from the ozonation of oleic acid, and since both of these are monounsaturated fatty acids, they would not be expected to undergo significant autoxidation. However, hexanal can arise from the ozonation of any n-6 unsaturated fatty acid or the autoxidation of any n-6 PUFA to a lipid hydroperoxide (Cuo et al., 1994). (Autoxidation of an n-6 PUFA can produce a hydroperoxide on the sixth carbon from the end of the chain; this can be reduced to the alkoxy radical, which can undergo β-scission to give hexanal.) Thus, hexanal is a marker of generalized oxidative stress as well as being an ozonation product.

For all three aldehydes, the largest apparent yields of aldehydes were observed after exposure for 60 min to 2.5 ppm ozone.
ozone with 5% CO₂. These maximum apparent yields were 184, 87, and 131 nM for hexanal, heptanal, and nonanal, respectively. (We will discuss pathways for the loss of aldehydes from lavage, and why these values are best viewed as apparent rather than absolute yields, in a subsequent section.) Figure 1A presents a plot of the 2.5 ppm ozone plus 5% CO₂, and shows that the apparent yields of the three aldehydes increase with time of exposure up to 60 min and then decrease.

The same pattern of increasing and then decreasing apparent yields of aldehydes can be observed with exposure to ozone alone, except that the highest apparent yields for hexanal and heptanal occur at 90 min (see Fig. 1B). The longer time required for the observance of the maximum apparent yield in the absence of CO₂ undoubtedly can be attributed to the smaller tidal volume of the exposures.

Dose-Response Data

Having established utility of the incorporation of CO₂ in the gas, other exposure levels of ozone were examined. The apparent yields of the three aldehydes measured in the BAL of rats exposed to 0.5 and 1.2 ppm ozone plus 5% CO₂ are shown in Figs. 2A and 2B, respectively. Again the pattern is an increasing apparent yield followed by a drop-off; for 0.5 ppm ozone plus 5% CO₂, aldehyde yields peak at about 90 min (see Fig. 2A).

In contrast to the data for 0.5 ppm (Fig. 2A) and 2.5 ppm (Fig. 1A), the results obtained for rats exposed to 1.2 ppm ozone plus 5% CO₂ (Fig. 2B) show that the aldehyde apparent yields continued to increase with the time of exposure up to 2 hr. We will discuss these patterns below.

Nonanal as an Ozone-Specific Biomarker

Figure 3 shows data for nonanal alone. It had been our expectation that nonanal, which is produced in the ozonation of the monounsaturated oleic acid, would be a relatively specific biomarker for ozone, since monounsaturated fatty acids do not undergo significant autoxidation. The apparent yields of nonanal in 1-hr exposures to ozone plus 5% CO₂ increase up to 2.5 ppm and then decrease; exposure to 5.0 and 10.0 ppm ozone without CO₂ leads to less nonanal being detected (37 and 50 nM, respectively) than 2.5 ppm ozone (73 nM).

Continued Detection of Aldehydes during the Recovery Time after Exposure

In order to determine the length of time the aldehydes could be detected in rat BAL after the termination of the ozone exposure, an experiment that allowed the rats to recover for 5, 18, and 24 hr after ozone exposure was performed (see Fig. 4). The largest apparent yields of aldehydes were found in rats exposed to 2.5 ppm ozone plus 5% CO₂ for 60 min, and, in order to have the biggest signal to follow, this exposure was chosen to measure the decrease in aldehydes with time. For rats euthanized as soon as possible
after exposure, the amount of hexanal, heptanal, and nonanal measured were 184, 88, and 131 nM, respectively. Five hours after exposure, these amounts decreased to 41 nM for hexanal, 8 nM for heptanal, and 21 nM for nonanal. At 18 and 24 hr after exposure only 4.3 ± 0.3 nM hexanal could be detected and both nonanal and heptanal were at background levels. The prolonged slight elevation in hexanal probably reflects the fact that ozone-induced lipid peroxidation of PUFA continues in the lungs of the exposed rats for hours after exposure and that hexanal measures generalized oxidation stress (Cueto et al., 1992).

**Apparent and Absolute Yields; Pathways for Loss of Lavage Aldehydes**

In the type of flow system that must be used in animal exposures, the moles of ozone that are deposited (i.e., react) in an animal lung is unknown, and it is not possible to express the data as absolute yields (i.e., moles of aldehydes produced per mole of ozone that reacted). In addition, there are a number of pathways that can be envisioned in which aldehydes could disappear from the compartments sampled by lavage, including volatilization of small aldehydes into the exhaled air; diffusion of aldehydes into tissues that are not well sampled by the lavage technique, particularly as ozone exposure continues and lung damage increases; and oxidation of aldehydes to acids (which are not detected by our method). (Ozone is known to initiate the air oxidation of aldehydes (Bailey, 1982).) Aldehydes also probably react with amine functionalities (e.g., in albumin) in the ELF to form Schiff bases; however, Schiff base formation is reversible, and the PFBHA method we used to derivatize the aldehydes converts them to the PFBHA oxime. (Data not shown.) Thus, Schiff base formation does not represent a loss pathway for these aldehydes.

Using an isolated rat lung, where the moles of ozone...
absorbed by the lung can be measured, we have found apparent yields of a particular aldehyde are obtained that equal about 0.1% mole per mole of ozone absorbed (Postlethwait et al., 1995). Since there are about 40 LOP that would be produced from the common unsaturated fatty acids in a rat lung (Pryor et al., 1995a, b), the total yield of all LOP in the BAL probably is in the range of 1–4%.

Rates of Aldehyde Production

Table 3 shows data on exposures to ozone plus 5% CO₂ recalculated as the nanomoles of aldehyde/form divided by the time period; the data are expressed as nm aldehyde/hr. (All of the data shown are compiled from Figs. 1A, 2A, 2B, and 4; standard deviations have been omitted from this table in order to make the trends easier to follow.)

Reading across Table 3 from left to right give the time dependence of the rate of production of a given aldehyde; for all three levels of ozone, 0.5, 1.2, and 5.0 ppm, the rate of production of all three of the aldehydes is a maximum at 0.5 hr and then decreases. For example, compare the situation after 1 and 2 hr of exposure. If twice as much of a given aldehyde were present at 2 hr compared with 1 hr, then the rate of production of that aldehyde would have remain unchanged. This is not what is observed; therefore, either the rate of production of LOP decreases with time or the rate of disappearance of LOP from the ELF increases with time, or both. It is likely that both of these factors occur. The rate of production of LOP could decrease with time since the rats may breathe less deeply with time during exposure. In addition, the leakage of compounds from the ELF into the subepithelial space and blood vessels undoubtably increases with the severity of the ozone exposure so that increasing amounts of aldehydes migrate from the ELF into compartments that are not sampled by the lavage technique. (In a few cases at the high exposure levels it was observed that the BAL appeared tainted with blood, suggesting increased leakage.) Leakage into the ELF also may raise the level of iron in the ELF, which could catalyze the oxidation of aldehydes to acids, which are not detected in our method.

SUMMARY, CONCLUSIONS, AND A LOOK FORWARD

LOP as Biomarkers for Ozone Exposure

This project was designed to discover biomarkers that reflect ozone exposure, and preferably those that could be used in humans. We selected lipid ozonation products as potential biomarkers for several reasons.

1. Unsaturated pulmonary lipids are known to undergo ozonation when animals inhale ozone (Rabinowitz and Bassett, 1988).

2. The primary molecular targets for ozone must be present in the first tissue layer that it passes through at the air/tissue boundary, and unsaturated fatty acids are present in both the lipids of the ELF and the membranes of the cells that line the airways (Pryor, 1992; Pryor et al., 1995b).

3. The products from the ozonation of unsaturated fatty
acids can be predicted from the Criegee ozonation mechanism. Only a small number of LOP are produced from any given unsaturated fatty acid, and the number of different unsaturated fatty acids present in lung lipids is quite small.

In fact, we are able to detect aldehydes in the lavage of rats and, in unpublished work, in humans (Squadrito et al., 1992a) that have been exposed to low levels of ozone for short times. These results prove, for the first time, that unsaturated pulmonary lipids, probably primarily in the ELF, do undergo ozonation in both rats and humans when ozone is inhaled and that the products from these reactions can be detected in BAL. Some authors had questioned whether the ozonation of unsaturated fatty acids could occur in competition with other reactive substances such as glutathione, ascorbate, and reactive proteins that are present in the lung (Freeman et al., 1979a,b; Banerjee and Mudd, 1992; Pryor and Uppu, 1993).

In our experiments, and also in environmental exposures, ozone is the limiting reagent; that is, the moles of reactive compounds in the ELF far exceed the moles of ozone inhaled. Therefore, relatively similar yields of LOP can be expected even for animals with slightly different concentrations of unsaturated fatty acids in the ELF. However, aldehydes are found in lavage only in very small amounts (Postlethwait et al., 1995). That, in combination with the fact that different animals have different ratios of lipids to other reactive species (such as ascorbate and glutathione) in the lung and different fatty acid profiles, suggests that these aldehydes probably are a rather imprecise dosimeter of ozone exposure. Thus, although these aldehydes appear to be excellent biomarkers of ozone exposure, quantitative dosimetry using aldehydes (and perhaps LOP in general) may be less satisfactory.

The Cascade Theory

Our identification of aldehydes, as well as other LOP, in lavage (Wright et al., 1994b; Kafoury et al., 1996; Cueto et al., 1992, 1994; Pryor et al., 1992), coupled with the recognition that ozone itself cannot penetrate far enough into the lung to cause many of the effects associated with the inhalation of ozone (Pryor, 1992), led us to recognize (Wright et al., 1994b; Pryor et al., 1995b) that LOP might be acting as signal transduction molecules, relaying the effects of ozone into deeper tissue strata. It is clear that the transmission of the damage by ozone requires a stable molecular species, and LOP appear to fill the bill. [Radicals are too unstable and exist only at very low concentrations (Pryor, 1994).] LOP disrupt the ordered structure of membranes and can cause the activation of lipases that can release inflammatory species (Salgo et al., 1994, 1995a).

The lowest ozone concentration studied here is 0.5 ppm; this relatively high level was necessary to detect the aldehydes we measured. However, the effects of ozone exposures in resting rats underestimate those observed in exercising humans (Hatch et al., 1994). In preliminary analyses of the BAL from humans exposed to 0.1 to 0.2 ppm ozone for 2–4 hr while exercising, exposures performed in the laboratory of Professors M. Utell and M. Frampton, we find that some of these aldehydes can be detected in amounts as great or greater than from rats exposed to 0.5 ppm ozone plus 5% CO₂ for 4 hr (unpublished data).

Further work on LOP should involve the demonstration that LOP signal the release of traditional inflammatory factors such as eicosanoids (Leikauf et al., 1993) and platelet-activating factor (Wright et al., 1994a; Pryor et al., 1995a). In addition, it is possible that LOP alter the activity of key enzymes in the lung that control eicosanoid and nitric oxide synthesis.

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