

Chemical Nature and Immunotoxicological Properties of Arachidonic Acid Degradation Products Formed by Exposure to Ozone

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Ozone (O₃) exposure *in vivo* has been reported to degrade arachidonic acid (AA) in the lungs of rodents. The O₃-degraded AA products may play a role in the responses to this toxicant. To study the chemical nature and biological activity of O₃-exposed AA, we exposed AA in a cell-free, aqueous environment to air, 0.1 ppm O₃, or 1.0 ppm O₃ for 30–120 min. AA exposed to air was not degraded. All O₃ exposures degraded >98% of the AA to more polar products, which were predominantly aldehydic substances (as determined by reactivity with 2,4-dinitrophenylhydrazine and subsequent separation by HPLC) and hydrogen peroxide. The type and amount of aldehydic substances formed depended on the O₃ concentration and exposure duration. A human bronchial epithelial cell line (BEAS-2B, S6 subclone) exposed *in vitro* to either 0.1 ppm or 1.0 ppm O₃ for 1 hr produced AA-derived aldehydic substances, some of which eluted with similar retention times as the aldehydic substances derived from O₃ degradation of AA in the cell-free system. *In vitro*, O₃-degraded AA induced an increase in human peripheral blood polymorphonuclear leukocyte (PMN) polarization, decreased human peripheral blood T-lymphocyte proliferation in response to mitogens, and decreased human peripheral blood natural killer cell lysis of K562 target cells. The aldehydic substances, but not hydrogen peroxide, appeared to be the principal active agents responsible for the observed effects. O₃-degraded AA may play a role in the PMN influx into lungs and in decreased T-lymphocyte mitogenesis and natural killer cell activity observed in humans and rodents exposed to O₃. **Key words:** aldehydes, arachidonic acid, BEAS cells, hydrogen peroxide, ozone, polarization. *Environ Health Perspect* 101:154–164(1993)

Exposure of humans to ozone (O₃; 0.1–0.6 ppm) for 2–6.6 hr can result in a lung inflammatory response as evidenced by an influx of polymorphonuclear leukocytes (PMN) into the airways (1–3). Additionally, a number of other immune cell functions can be altered upon O₃ exposure. For example, a depression in mitogen-stimulated peripheral blood T-lymphocyte proliferation was observed in humans exposed to 0.4–0.6 ppm O₃ for 2–4 hr (4–6). O₃ exposure *in vitro* of human peripheral blood mononuclear cells inhibited T-lymphocyte proliferation and decreased interleukin-2 and IgG production (7). Human peripheral blood natural killer (NK) cells exposed *in vitro* to 0.18–1.0 ppm O₃ for 4 hr demonstrated decreased cytotoxicity against K562 tumor cells (8). Rodents exposed *in vivo* to 0.5–1.0 ppm O₃ for 1 day (9) or 1.6 ppm for 7 days (10) also demonstrated decreased lung NK activity.

Arachidonic acid (AA) is a polyunsaturated fatty acid that is found in lung cells and in airway lining fluid either free or esterified to phospholipids (11). We have previously shown that in a cell-free *in vitro* exposure system, O₃ can degrade AA to more polar products (12). Additionally, exposure of rats to 2.0 ppm O₃ *in vivo* for 4 hr has been reported to degrade AA in the lung (13). The products of O₃-induced degradation of fatty acids in aqueous solutions have been reported to include carbonyl compounds (aldehydes and ketones), ozonides, and peroxides (14–16).

Some of the observed responses of immune cells to O₃ exposure *in vivo* and *in vitro* (e.g., neutrophil influx, decreased lymphocyte proliferation, suppressed NK cytotoxicity) may be mediated by O₃-induced AA degradation products. For example, long-chain aldehydes have been shown to be potent chemoattractant agents for rat polymorphonuclear leukocytes (PMNs) (17,18). Hydrogen peroxide (H₂O₂) has been reported to inhibit human peripheral blood T-lymphocyte proliferation (19) and NK cell lysis of K562 tumor cells (20). We therefore examined whether the degradation products of AA formed by O₃ exposure *in vitro* possessed

biological activity using assays that assess PMN polarization (which is an indicator of the chemokinetic and/or chemotactic potential of a substance), PMN superoxide anion (O₂⁻) production, lymphocyte proliferation, and NK cytotoxicity. We also examined the chemical nature of the O₃-induced AA degradation products and the effect of O₃ exposure *in vitro* on the formation of these products by a human bronchial epithelial cell line.

Methods

Materials

AA (sodium salt; 99% purity), catalase (bovine liver), superoxide dismutase (SOD; bovine erythrocyte), *N*-formyl-methionine-leucine-phenylalanine (fMLP), H₂O₂, reduced glutathione (GSH), GSH peroxidase (bovine erythrocyte), ferricytochrome c (horse heart), 2,4-dinitrophenylhydrazine (DNPH), dextran sulfate (500,000 average molecular weight), and phenol-red-free Hank's balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ were purchased from Sigma Chemical Company (St. Louis, MO). ³H-AA (60–100 Ci/mmol, >98% *cis* form, tritium attached to carbon in positions 5,6,8,9,11,12,14, and 15) and ³H-thymidine (20 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Phosphate-buffered saline (PBS; Ca²⁺ and Mg²⁺ free) and RPMI medium were purchased from Hazelton Laboratories (Lexena, KS) or JRH Biosciences (Lexena, KS). Ficoll-Hypaque was purchased from Organon-Teknika (Durham, NC). Keratinocyte growth medium was purchased from Clonetics Corp. (San Diego, CA). Six- (35-mm² diameter/well) and 96- (0.32 cm²/well) well plastic tissue culture plates were purchased from Costar (Cambridge, MA). Phytohemagglutinin (PHA) and concanavalin A were purchased from Burroughs-Wellcome (Research Triangle Park, NC).

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Exposure of AA Solutions to O₃

AA (30 μM) in 500 μl PBS, or PBS alone, was added to 35-mm wells and exposed to air or O₃ (0.1 or 1.0 ppm) for up to 120 min. We chose these O₃ concentrations to examine the AA degradation products formed with very low and very high concentrations of the oxidant gas. We maintained O₃ concentrations within ± 10% and ± 20% of the 1.0 ppm and 0.1 ppm concentrations, respectively. In some experiments requiring radioactive AA as a tracer compound, ³H-AA in ethanol was added to a polypropylene tube and the ethanol evaporated under N₂ (37°C). We then added the unlabeled 30 μM AA solution to the ³H-AA (0.2 Ci/ml final concentration; approximately 2.5 nM), sonicated the solution (30 sec) in a water bath sonicator (Fisher Scientific), and vigorously agitated the solution before exposure to air or O₃. Exposures were performed in a humidified rocking *in vitro* exposure system (37°C, 95% relative humidity, 5% CO₂) previously described in detail (7, 20, 21). Room air was filtered through a high-efficiency particle air filter (to remove particulates) and an activated charcoal filter (to remove gaseous pollutants) and was pumped into the air (no O₃) exposure chamber or was passed through a metal chamber containing a UV light source that produced O₃. Oxides of nitrogen were not detectable in the exposure chamber atmosphere. After exposure, solutions were removed from the exposure chambers and used in cell bioassays or analyzed for hydroperoxides and/or carbonyl compounds. In studies designed to examine the reactivity of O₃-exposed AA with GSH, solutions of O₃-exposed AA (900 μl) were incubated (60 min, 37°C) with GSH (100 μl in PBS with pH adjusted to 7.4; final GSH concentrations of 1–100 mM) or 10 mM GSH with GSH peroxidase (5 U/ml). In some experiments, we incubated solutions (60 min, 37°C) with catalase (120 U/ml) and SOD (107 U/ml) and/or ethanol (1 mM) to scavenge H₂O₂, O₂⁻, and hydroxyl radical, respectively, before adding the solutions to bioassays. The recovery of tritium derived from ³H-AA in the air-exposed and O₃-exposed wells was >50% and >70%, respectively. The radioactive solutions were then analyzed for the presence of carbonyl-containing compounds by HPLC.

HPLC Analysis of Carbonyl Compounds

We incubated solutions (500 μl) containing ³H-AA that had been previously exposed to air or O₃ for 60 min in the dark with 100 μl HCl (12 M) and 500 μl acetonitrile with or without DNPH (0.125%). DNPH has been reported to selectively

react with carbonyl moieties, i.e., aldehydes and ketones (22). Aliquots of each sample were then injected into an HPLC system which consisted of two 510 pumps, a WISP 712 autosampler, a Systems Interface Module, and a Maxima 820 software program (Waters' Associates, Milford, MA). We separated tritiated substances using a modified version of a previously published reverse-phase method (23) and a gradient from 60/40 acetonitrile/ water to 100% acetonitrile over 45 min at 1.0 ml/min flow rate through a Beckman/Altex 5 μM ODS silica column (Rainin Instruments, Woburn, MA). For the purposes of this report we have termed this HPLC system 1. Eluting radioactivity was monitored using a flow-through scintillation counter (Radiomatic Instruments, Tampa, FL) pumping scintillation cocktail at 3.0 ml/min with automatic quench correction of the radioactivity.

Peroxide Analyses

The amount of lipid peroxides in O₃-exposed AA solutions or media from O₃-exposed BEAS cells was determined using a commercial kit (Determiner LPO, Kamiya Biomedical Company, Thousand Oaks, CA) that measures the formation of methylene blue via the hemoglobin-catalyzed reaction of a methylene blue derivative (10-*N*-methylcarbamoyl-3,7-dimethylamino-10-hydroxyphenothiazine; MCDP) with lipid peroxides. Formation of methylene blue was monitored spectrophotometrically at 660 nm, and cumene hydroperoxide was used as a standard. H₂O₂ (50 μM) did not react with MCDP. We determined H₂O₂ using the horseradish peroxidase-catalyzed oxidation of phenol red, indicated by a change in absorbance at 610 nm (24).

Exposure of a Human Bronchial Epithelial Cell Line to Ozone

Characteristics of BEAS-2B cells (S6 subclone; hereafter referred to as BEAS cells) have been previously described (25,26) and were generously provided by Curtis Harris and John Lechner (National Institutes of Health, Bethesda, MD). Briefly, human bronchial epithelial cells were transformed with an Ad12-SV40 construct. This cell line is differentiation sensitive to transforming growth factor and serum, can produce mucuslike glycoproteins, and contains cytokeratin. We seeded BEAS cells (passages 79–99) at 5 × 10⁵ cells on collagen-coated filters (24 mm diameter; 3 μm pores) in a Transwell-COL system (Costar, Cambridge, MA) with 1.0 ml and 2.0 ml keratinocyte growth medium in the apical and basolateral compartments, respectively, as previously reported (27). We changed media on day 2 of culture, and then on day

4 of culture, we added 2 μCi ³H-AA in 1.0 ml keratinocyte growth medium to each well in the apical compartment. On day 5, we removed unincorporated ³H-AA, washed cells twice with HBSS, and exposed cultures to air or O₃ (0.1 or 1.0 ppm) for 60 min with 1.6 ml HBSS in the basolateral compartment, using the same *in vitro* exposure system as described previously but without rocking. At the end of the exposure, cells were removed from the exposure system, 1.0 ml HBSS was added to the apical compartment, and the cells were incubated an additional 5 min at 37°C and 5% CO₂. We then removed conditioned media (keeping the apical and basolateral compartment media separate from each other), centrifuged the media (300g, 5 min, 4°C), added the supernatants to ethanol (80% alcohol final concentration), and cooled the mixture (-80°C). Precipitated proteins were removed by centrifugation (500g, 20 min, 10°C), the supernatants were dried by Speed Vac evaporation (Savant, Farmingdale, NY) and redissolved in 500 μl 5% methanol initially, and then the tube was reextracted with 500 μl 95% methanol, and the extracts were combined (tritium extraction efficiency >65%). We incubated 450 μl of the supernatant with acidified DNPH and an equal volume with vehicle (acidified acetonitrile) in a similar manner as the O₃-exposed AA solutions. The solutions were injected into the HPLC system described previously but using different separation conditions (28) that employed a reverse-phase methanol–water–acetic acid gradient separation over 100 min. We term this second separation system HPLC system 2 for the purposes of this report.

Peripheral Blood Leukocyte Isolation

Human peripheral blood cells were initially separated using a standard Ficoll-Hypaque gradient separation technique (29). For isolation of PMN, we removed cells from the appropriate layer, added them to 6% dextran (in PBS) for 45 min, and removed the top layer containing the PMN. Residual erythrocytes were lysed twice in 5 ml ice-cold, sterile distilled water, and isotonicity was restored with an equal volume of 1.8% saline and then 40 ml PBS after each hypotonic lysis. PMN viability was 95% as assessed by the trypan blue dye exclusion assay, and there was <5% erythrocyte contamination. For isolation of T-cell lymphocytes, we removed the mononuclear cell layer from the Ficoll-Hypaque gradient, and most monocytes were removed by adherence to plastic tissue culture dishes (1 hr at 37°C in 5% CO₂). We then used nonadherent cells for T-cell proliferation assays. For isolation of NK cells, the nonadherent mononuclear

cells were passed through nylon wool columns (30) and the nonadherent cells collected for NK assays.

Cell Bioassays

Polarization was assessed using a modification of a previously published method (31). PMN were adjusted to 1×10^6 cells/ml in HBSS. We added 900 μ l of the cell suspension to 100 μ l of a test solution (resulting in a 1/10 dilution of the test solution) and incubated it at 37°C for 10 min with shaking. Cells excluded >95% trypan blue after the incubation period with all solutions tested. Cells were then fixed by the addition of 1.0 ml ice-cold 3.7% (v/v) formaldehyde in PBS. We counted a minimum of 200 cells for polarization by standard light microscopic techniques. Duplicate variability averaged <9%. To confirm that the PMN had undergone a shape change in the presence of a test solution, the forward-angle light scatter of a sample was determined using standard flow cytometric techniques using an Epics 5 Counter (Coulter Corp., Hialeah, FL) with a 1–19° fixed angle of light collection. Human blood PMN have been shown to have a decrease in forward-angle light scatter when exposed to chemotactic agents (32).

We measured PMN O_2^- formation using the SOD-inhibitable reduction of ferricytochrome c (33). We incubated PMN (1×10^5) in 490 μ l HBSS with 250 μ l of a test solution, 250 μ l of ferricytochrome c (final concentration of 80 μ M), and 10 μ l SOD (320 U/ml final concentration) or 10 μ l HBSS in polypropylene tubes at 37°C with shaking for 60 min. In some experiments, PMN were preincubated with O_3 -exposed AA solutions for 60 min before the addition of stimuli. Tubes were then centrifuged (500g, 5 min, 4°C), and the absorbance of the supernatant was measured at 550 nm.

To assess T-lymphocyte proliferation, we incubated 1×10^6 nonadherent cells/ml PBS in polypropylene tubes for 30–60 min (37°C, 5% CO_2) with an equal volume of a test solution that had been previously filtered (0.22 μ m) to prevent microbial contamination of the cell cultures. In separate experiments, recovery of the O_3 -exposed AA after filtration was >80% based on the percentage of O_3 -exposed 3H -AA that passed through the filter. Cell viability after the preincubation period was $92 \pm 2\%$, $77 \pm 7\%$, and $80 \pm 4\%$ for cultures incubated with PBS alone, O_3 -exposed AA (1.0 ppm \times 120 min), and O_3 -exposed PBS (1.0 ppm \times 120 min), respectively ($p = NS$ among the three groups). After the preincuba-

tion period, 100- μ l aliquots of the cell suspension were transferred to a 0.32-cm² well, and 100 μ l RPMI media with gentamicin (40 μ g/ml) and 20% fetal calf serum and 50 μ l RPMI with 10% fetal calf serum containing either concanavalin A (5 μ g/ml final concentration) or PHA (0.6 μ g/ml final concentration) or vehicle alone were added. We assayed samples in quadruplicate. Cultures were incubated for 72 hr, and then proliferation was assessed by 3H -thymidine incorporation for 18 hr as previously described (7).

To assess NK cytotoxicity, nonadherent lymphocytes (2.5×10^6 /ml PBS) were incubated with an equal volume of a test sample for 1 hr (37°C, 5% CO_2) and centrifuged (500g, 5 min, 23°C). We then aspirated the supernatant and resuspended the cells in RPMI with 10% fetal calf serum. The NK activity of the cells was assessed in a standard 4-hr cytotoxicity assay against K562 target cells using effector:target ratios from 50:1 to 1.8:1 as previously described (34). We assayed all samples in triplicate, and data are expressed as lytic units at the LU_{10} value (10% specific cytotoxicity level) calculated by regression analysis (35).

The effect of O_3 -exposed AA on the ability of NK cells to bind to K562 target cells was determined using a modification of a previously published method (36). We added 0.2×10^6 NK cells to an equal number of K562 cells in a total volume of 400 μ l RPMI with 10% fetal calf serum, centrifuged the cells (300g, 5 min, 23°C), and removed 200 μ l supernatant. Cells were incubated for 15 min (37°C, 5% CO_2), and then cells were pipetted three times to disrupt spontaneous conjugate formation. We then determined cell conjugates in duplicate by standard light microscopic enumeration.

All data are expressed as means \pm SEM. A t -test for paired variates was used for all analyses. Values of $p < 0.05$ are considered significantly different (37).

Results

Products of O_3 -exposed AA

AA (containing 3H -AA) was exposed to air or O_3 , incubated with acidified DNPH or vehicle, and then analyzed by HPLC (system 1). AA exposed to air for 120 min

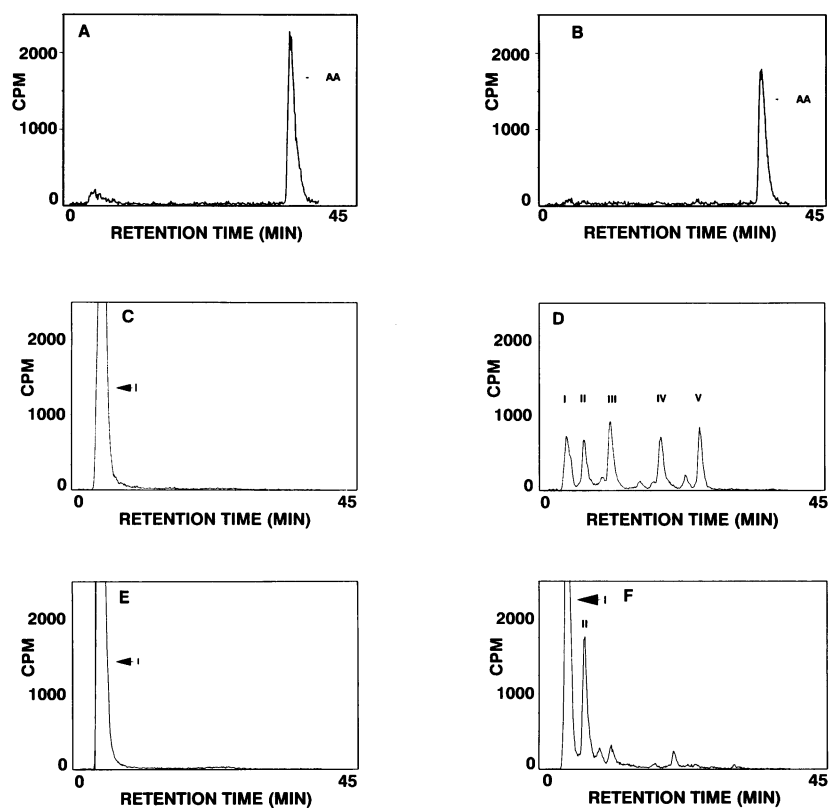


Figure 1. HPLC radioactivity profile of 2,4-dinitrophenylhydrazine (DNPH)-reactive products of 3H -arachidonic acid (AA) exposed to air or O_3 . AA (30 μ M) in phosphate-buffered saline with 3H -AA tracer present was exposed to air or O_3 , the solutions were removed, incubated with DNPH or vehicle (acidified acetonitrile), and injected into HPLC system 1. See text for more details. AA exposed to air (120 min) and incubated with (A) vehicle or (B) DNPH. AA exposed to 0.1 ppm O_3 for 30 min and incubated with (C) vehicle or (D) DNPH. AA exposed to 1.0 ppm O_3 for 120 min and incubated with (E) vehicle or (F) DNPH. Chromatograms are typical for $n \geq 5$ separate experiments.

and then incubated with vehicle only eluted with a retention of approximately 38 min (Fig. 1A). Air-exposed AA incubated with DNPH eluted with a similar retention time (Fig. 1B). In contrast, almost all of the radioactivity associated with O₃-exposed AA (0.1 ppm for 30 min) that was incubated with vehicle eluted in the HPLC system as a polar peak with an approximate retention time of 4.5 min (Fig. 1C), suggesting that O₃ degraded AA. HPLC chromatograms of the 0.1 ppm O₃-exposed AA that was incubated with DNPH demonstrated the presence of five major peaks (peaks I–V; Fig. 1D). Therefore, O₃-exposed AA (0.1 ppm for 30 min) reacted with DNPH as evidenced by a shift in retention times of some of the radioactivity in the chromatograms and as evidenced by the fact that these products contain carbonyl moieties. Chromatograms of 1.0 ppm O₃-exposed AA (120 min) incubated with vehicle (Fig. 1E) showed the presence of a polar, degraded AA peak that eluted with a similar retention time as the 0.1 ppm O₃-degraded AA solution. The chromatograms of 1.0 ppm O₃-AA (120 min) incubated with DNPH (Fig. 1F) showed the presence of two major peaks (I and II), of which peak II had a different retention time from the radioactivity exhibited in the chromatograms of vehicle-incubated solutions. These chromatographic data show that the quantity and types of products in the 1.0 ppm O₃-AA solution (120 min) were different from the products in the 0.1 ppm (30 min) O₃-AA solutions.

The chemical stability of the carbonyl compounds was assessed next. AA, exposed to 0.1 ppm for 30 min, was either immediately incubated with DNPH after exposure or left at 22°C for 24 hr in the dark (in a tightly capped polypropylene tube) before incubation with DNPH. Analysis of the resulting products by HPLC was performed immediately after DNPH incubation. HPLC analysis showed that there was <16% decrease in the radioactivity associated with peaks II–V, with a slight increase (5%) in the radioactivity associated with the peak I (data not shown).

The ability of the antioxidant GSH to react with the O₃-exposed AA was then tested. AA (exposed to 0.1 ppm O₃ for 30 min) that was preincubated with vehicle (0 mM GSH) or 1 mM GSH and then reacted with DNPH produced five major peaks (Fig. 2A, B). Incubation of O₃-exposed AA with 10 mM GSH decreased the radioactivity 38%, 29%, and 36% in peaks III, IV, and V, respectively, with peak I increasing 220% and peak II remaining unchanged (Fig. 2C). Incubation of O₃-exposed AA with 100 mM GSH removed

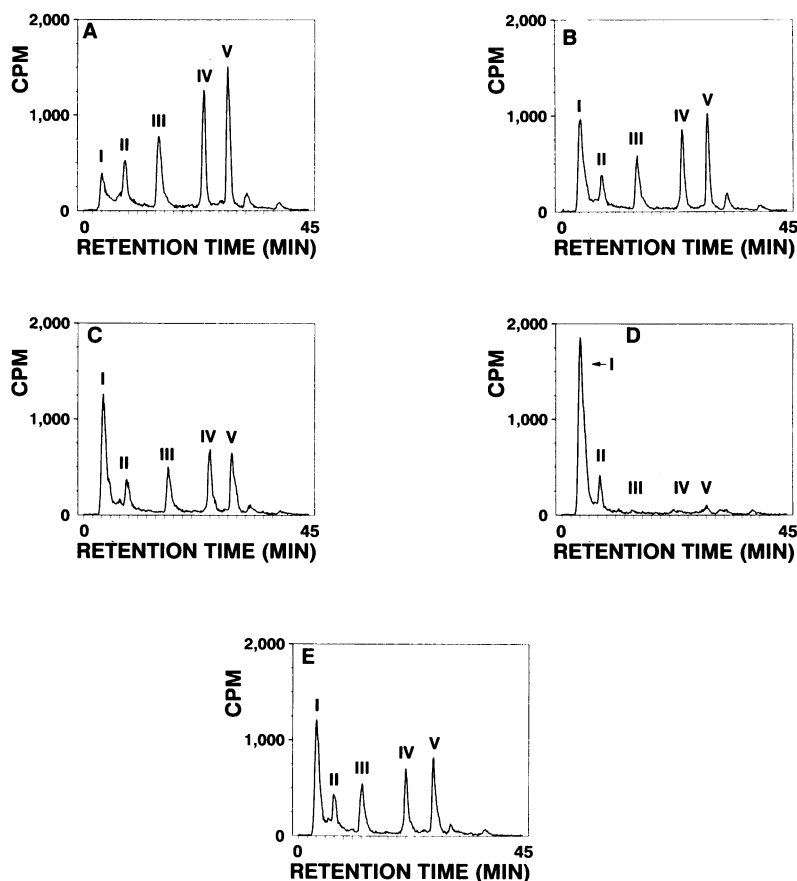


Figure 2. Effect of glutathione (GSH) on chromatographic retention of 2,4-dinitrophenylhydrazine (DNPH) derivatives of O₃-degraded arachidonic acid (AA). AA (30 μM in phosphate-buffered saline with ³H-AA tracer) was exposed to 0.1 ppm O₃ for 30 min, solutions incubated with 0, 1, 10, or 100 mM GSH or 10 mM GSH and GSH peroxidase (60 min at 37°C), and then solutions were incubated with DNPH (60 min) before injection into HPLC system 1. Typical chromatograms are shown. (A) O₃-exposed AA incubated with 0 mM GSH (*n* = 3), (B) 1 mM GSH (*n* = 2), (C) 10 mM GSH (*n* = 3), (D) 100 mM GSH (*n* = 3), and (E) 10 mM GSH plus GSH peroxidase (5 U/ml) (*n* = 2).

almost all of peaks III, IV, and V (88%, 94%, and 89% decrease, respectively) with a concomitant 555% increase in radioactivity in peak I and no change in peak II (Fig. 2D). Incubation of the O₃-degraded AA with both 10 mM GSH and GSH peroxidase (5 U/ml) did not reduce peaks III–V beyond that seen with 10 mM GSH alone (Fig. 2E). In separate experiments, 100 mM GSH did not react with DNPH. Therefore, the decrease in peaks III–V upon incubation of O₃-exposed AA with 10 or 100 mM GSH was not due to a GSH-induced interference with the reaction of DNPH with carbonyl groups.

Exposure of either PBS or AA (30 μM in PBS) to air for up to 120 min resulted in the production of H₂O₂ at concentrations <4 μM (Table 1). PBS exposed to 0.1 ppm O₃ (for 30 min) resulted in the production of a low amount of H₂O₂. Exposure of PBS to a higher concentration and duration of O₃ (1.0 ppm for 120 min) increased the H₂O₂ concentration to 8.7 μM. In contrast, exposure of AA to 0.1 ppm O₃ (30 min) resulted in an H₂O₂ concentration that was 9.4-fold higher

than that found with the O₃ exposure of PBS alone (20.8 μM versus 2.2 μM H₂O₂; *p* < 0.05). Similarly, exposure of AA to 1.0 ppm for 120 min resulted in a 5.6-fold higher concentration of H₂O₂ than compared to O₃ exposure of PBS alone (48.9 μM versus 8.7 μM; *p* < 0.05). Less than 0.1 μM (cumene hydroperoxide-equivalents) of organic peroxide was detected upon incubation of the O₃-exposed AA (0.1 ppm for 30 min or 1.0 ppm for 120 min) with MCDP.

Formation of AA-derived Aldehydes by BEAS-S6 Cells

To examine if bronchial epithelial cells, cell types that are likely to be exposed to O₃, could also produce AA-derived aldehydic substances in response to O₃ exposure, we prelabeled BEAS cells with ³H-AA and exposed them to air or O₃. The conditioned media supernatants (apical and basolateral supernatants that were kept separate) from air and O₃-exposed BEAS-S6 cells were then incubated with either DNPH or vehicle alone. HPLC analysis (system 2) demonstrated that air-exposed

Table 1. Effect of O₃ on H₂O₂ concentration of phosphate-buffered saline (PBS) and arachidonic acid (AA) solutions

Exposure	Solution	Exposure time (min)	Concentration H ₂ O ₂ (μM)
Air	PBS	30	1.0 ± 0.4
	AA	30	1.0 ± 0.6
Air	PBS	120	1.9 ± 1.2
	AA	120	3.8 ± 0.6
O ₃ 0.1 ppm	PBS	30	2.2 ± 0.8
	AA	30	20.8 ± 4.6*
O ₃ 1.0 ppm	PBS	120	8.7 ± 1.3
	AA	120	48.9 ± 8.8*

PBS or AA (30 μM in PBS) were exposed to air or O₃ either for 30 or 120 min. After exposure, solutions were removed from the chambers, and H₂O₂ concentrations were determined by the horseradish peroxidase-mediated oxidation of phenol red. Values represent three separate experiments.

*Significant difference from paired O₃-exposed PBS value.

cells released primarily ³H-AA (at 83 min) and ³H-phospholipids (in the solvent front) into the basolateral compartment (Fig. 3A) and into the apical compartment (data not shown). This radioactivity did not shift retention time when incubated with DNPH. In contrast to the air-exposed cells, BEAS cells exposed to 0.1 ppm O₃ (1 hr) had an increased release of ³H products into the basolateral compartment, with the largest product being a relatively polar peak that eluted early in the HPLC run (Fig. 3B). Incubation of the supernatant from the basolateral compartment of 0.1 ppm O₃-exposed BEAS cultures with DNPH resulted in a shift of some tritium into products that eluted at a retention time of approximately 63 and 83 min. A similar profile of ³H-AA products was released into the apical compartment by 0.1 ppm O₃-exposed cells, but no increased radioactivity in any peaks was observed in the apical supernatant incubated with DNPH (data not shown). Similar to the 0.1 ppm O₃-exposed cultures, 1.0 ppm O₃-exposed BEAS cells also released a radioactive polar peak as the major product into the basolateral compartment (Fig. 3C). In contrast to the 0.1-ppm exposure group, incubation of the basolateral supernatant from 1.0 ppm O₃-exposed cells with DNPH resulted in a shift of tritium into several peaks that eluted at 26, 42, 63, 66, and 83 min. The polar peak also was the largest peak released into the apical compartment of 1.0 ppm O₃-exposed cells, and incubation of apical supernatants with DNPH also caused a shift in radioactivity with increased peaks at 26, 63, and 83 min (data not shown). The retention times of the five major peaks formed by O₃-exposed AA (0.1 ppm for 30 min) when reacted with DNPH and separated in HPLC system 2 are shown in Figure 3D as a reference. Some of the O₃-degraded AA peaks formed in the cell-free system that reacted with DNPH (i.e., 63 and 83 min retention) had similar retention times as DNPH-reactive products formed by 1.0 ppm O₃-exposed BEAS cells.

Because ³H-AA elutes at 83 min in HPLC system 2, we performed experiments to eliminate the possibility that the increased radioactivity that eluted at 83 min upon incubation of the supernatants from 0.1 ppm and 1.0 ppm O₃-exposed BEAS cells with DNPH was not due to an increased recovery of ³H-AA. ¹⁴C-AA was added to the supernatants from 1.0 ppm O₃-exposed cultures just before incubation with either DNPH or vehicle and then injected into the HPLC system. Recovery of the ¹⁴C-AA from the supernatants incubated with or without DNPH was 29,471 dpm and 29,389 dpm in the apical compartment extracts, respectively, and 27,524 dpm and 28,994 dpm in the basolateral compartment extracts, respectively. These data imply that 1) radioactive AA is not recovered in increased quantities upon incubation with DNPH and 2) the in-

creased radioactivity at 83 min in the BEAS media that reacted with DNPH was aldehydic in nature.

BEAS cells exposed to air or O₃ (either 0.1 or 1.0 ppm) for 60 min had nondetectable concentrations of lipid peroxides (<0.1 μM cumene hydroperoxide-equivalents) in the media of both the apical and basolateral compartments (data not shown; *n* = 2). H₂O₂ concentrations in the media of air-exposed and O₃-exposed BEAS cells were < 1 μM and < 2 μM, respectively (*n* = 4; *p* = NS).

In vitro Assessment of Biological Activity of O₃-exposed AA

To assess the chemotactic/chemokinetic property of the degradation products of O₃-exposed AA, the ability of O₃-exposed AA to induce human PMN polarization (shape change) was studied. Incubating PMN with PBS for 10 min polarized 15 ± 2% of the cells (Table 2). Incubation of PMN with fMLP (100 nM, final concentration) as a positive control increased the number of cells undergoing polarization to 79 ± 2% (*p* < 0.05 compared to PBS-incubated controls). AA exposed to 0.1 ppm O₃ for 30 min induced polarization of 50 ± 5% of the PMN (*p* < 0.05). AA exposed to 1.0 ppm O₃ (for 120 min) induced polarization of 24 ± 5% PMN (*p* = NS). PBS exposed to air or O₃ (0.1 or 1.0 ppm) for up to 120 min did not induce an increase in PMN polarization. PMN incubated with O₃-exposed AA (either 0.1 or 1.0 ppm) were not aggregated, and conse-

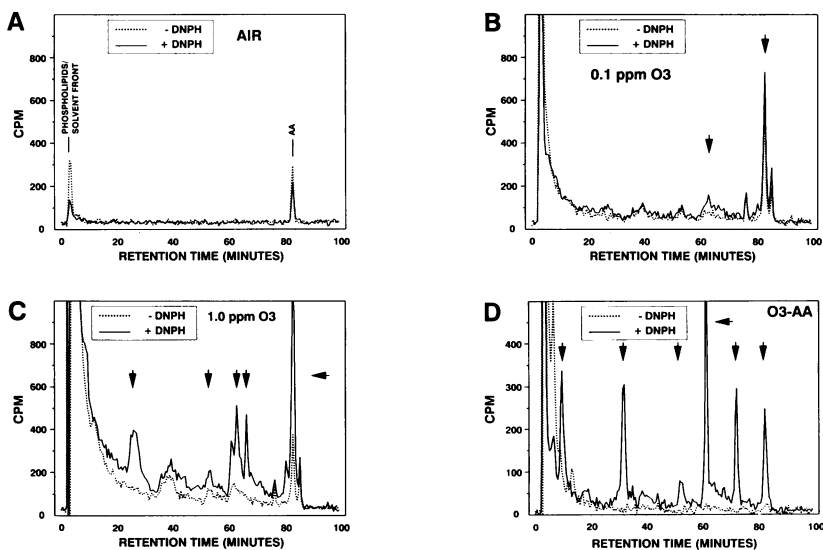


Figure 3. Effect of air or O₃ exposure on the formation of ³H-arachidonic acid (AA)-derived aldehydic products by BEAS-S6 cells. BEAS-S6, prelabeled with ³H-AA, were exposed to air or O₃ for 60 min in a Transwell system with Hank's balanced salt solution present in the basolateral compartment but not in the apical compartment. Media were removed, extracted, and incubated with 2,4-dinitrophenylhydrazine (DNPH) or vehicle before injection into HPLC system 2. See text for further details. Arrows indicate peaks of increased radioactivity in media incubated with DNPH compared to media incubated with vehicle alone. (A) Basolateral media extract of cultures exposed to air. (B) Basolateral media extract of cultures exposed to 0.1 ppm O₃. (C) Basolateral media extract of cultures exposed to 1.0 ppm O₃. (D) AA solution (30 μM in phosphate-buffered saline with ³H-AA tracer) exposed to 0.1 ppm O₃ for 30 min.

Table 2. Effect of O₃-exposed arachidonic acid (AA) on human PMN polarization

Exposure	Solution	Time (min)	% Polarization	n
None	PBS	—	15 ± 2	16
Air	PBS	30	14 ± 4	9
None	100 nM fMLP	—	79 ± 2*	16
O ₃ , 0.1 ppm	AA	30	50 ± 5*	13
	PBS	30	14 ± 2	15
O ₃ , 1.0 ppm	AA	120	24 ± 13	8
	PBS	120	19 ± 10	9

Abbreviations: PMN, polymorphonuclear leukocytes; PBS, phosphate-buffered saline; fMLP, *N*-formyl-methionine-leucine-phenylalanine.

AA (30 μM in PBS) or PBS was exposed to air or O₃ for the indicated times. PMN (9 × 10⁵ in 0.9 ml Hank's balanced salt solution) were incubated with solutions (100 μl) for 10 min at 37°C with rocking, then formalin was added. Cells were then examined by standard light microscopy counting techniques.

*Significant difference from PBS-incubated control cells, *p* < 0.05

quently cell aggregates did not interfere with determining polarization and forward-angle light scatter.

Forward-angle light scatter was measured to further confirm the effect of O₃-exposed AA on inducing a shape change in PMN. Typical forward-angle light scatter intensity data histograms are shown in Figure 4. PMN incubated with 100 nM fMLP had a 22 ± 3 channel decrease in mean channel forward-angle light scatter compared to cells incubated with PBS

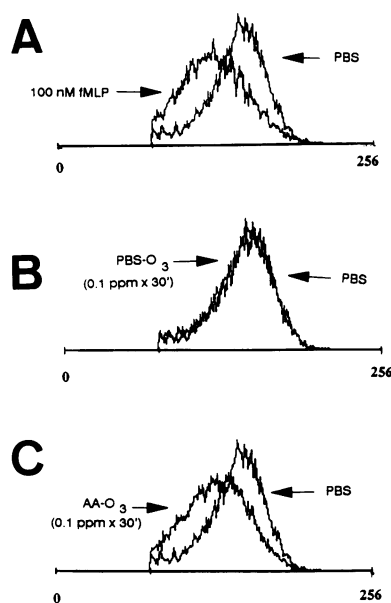


Figure 4. Forward-angle light scatter (FALS) flow cytometric analysis of human polymorphonuclear leukocytes (PMN) incubated with O₃-exposed solutions. PMN were incubated (10 min, 37°C) with phosphate-buffered saline (PBS), *N*-formyl-methionine-leucine-phenylalanine (fMLP; 10⁻⁷ M final concentration), O₃-exposed PBS (0.1 ppm for 30 min), or O₃-exposed AA (0.1 ppm for 30 min) and then fixed in formaldehyde. Cells were then analyzed by flow cytometry for changes in FALS. See text for more details. PMN incubated with (A) fMLP, (B) O₃-exposed PBS, and (C) O₃-exposed AA. Flow cytometric FALS of PMN incubated with PBS as a control are shown in each figure as a reference population. Data are typical of *n* = 4 independent experiments.

alone (*p* < 0.01). PMN incubated with O₃-exposed PBS (0.1 ppm for 30 min) had a 4 ± 3 channel decrease in mean channel forward-angle light scatter intensity (*p* = NS). However, PMN incubated with AA that was previously exposed to 0.1 ppm O₃ for 30 min had a decrease in mean channel forward-angle light scatter intensity of 17 ± 2 (*p* < 0.01).

The increase in PMN polarization induced by O₃-exposed AA (0.1 ppm for 30 min) could have been caused by several of the components. It may be possible that residual (nondegraded) AA, various oxygen species, and/or aldehydic substances contributed to the increased PMN polarization. Pretreatment of O₃-exposed AA (0.1 ppm for 30 min) with catalase, SOD, and ethanol before the addition of the solution to PMN did not attenuate the increased polarization (Table 3). H₂O₂ (3 mM; a concentration relevant to the amount produced during AA degradation by 0.1 ppm O₃; Table 1) by itself did not induce increased polarization. Nondegraded AA can compose up to 2% of the radioactivity seen in HPLC chromatograms of O₃-exposed AA. Incubation of

PMN with a similar concentration of AA that was recovered after an O₃ exposure (30 nM final concentration) did not increase PMN polarization. At 3 μM, AA alone increased PMN polarization to 61 ± 9%. Preincubation of PMN with both indomethacin and nordihydroguaretic acid (NDGA; 10 mM each, 15 min, 37°C) reduced the polarization induced by 3 μM AA to 28 ± 11%, suggesting that AA must be metabolized by cyclooxygenase and/or lipoxygenases to induce polarization. However, PMN preincubated with indomethacin and NDGA (to decrease the metabolism of residual AA) had similar polarization upon incubation with O₃-degraded AA (50 ± 16%; *p* < 0.05) compared with untreated PMN incubated with O₃-degraded AA (47 ± 8%). These data suggest that any residual AA in the O₃-exposed AA was not sufficient to increase PMN polarization and that the O₃-degraded AA products, unlike authentic AA, did not need to be metabolized by cyclooxygenase and/or lipoxygenases to induce polarization.

Pretreatment of O₃-exposed AA with 100 mM GSH (which eliminated most of the DNP-reactive peaks III–V; Fig. 2D) before addition to PMN resulted in a polarization value (4 ± 1%) similar to PBS control values (10 ± 4%; *p* = NS; Fig. 5). Incubation of the O₃-exposed AA with 10 mM GSH with or without GSH peroxidase (which partially eliminates peaks III–V) had no effect on the increase in PMN polarization. Pretreatment of PBS with 100 mM GSH had no effect on PMN polarization (*p* = NS).

Production of O₂⁻ by PMN in the presence of various test solutions was assayed. PMN (2 × 10⁵) incubated with phorbol myristate acetate (PMA; 100 ng/ml) produced 9.6 ± 1.6 nmol O₂⁻ (in 1 hr), which

Table 3. Effect of O₃-exposed arachidonic acid (AA) components on PMN polarization

Solution	Preincubation		%Polarization	n
	Solution	PMN		
PBS	None	None	15 ± 3	6
fMLP, 100nM	None	None	81 ± 3*	6
O ₃ -AA	None	None	47 ± 8*	6
PBS	CAT+SOD+EtOH	None	27 ± 5	3
O ₃ -AA	CAT+SOD+EtOH	None	56 ± 10*	3
H ₂ O ₂ 3 μM	None	None	16 ± 1	4
AA, 30 nM	None	None	18 ± 4	3
AA, 3 μM	None	DMSO	61 ± 9*	3
AA, 3 μM	None	INDO+NDGA	28 ± 11	3
O ₃ -AA	None	INDO+NDGA	50 ± 16*	3

Abbreviations: PMN, polymorphonuclear leukocytes; PBS, phosphate-buffered saline; fMLP, *N*-formyl-methionine-leucine-phenylalanine; CAT, catalase; SOD, superoxide dismutase; EtOH, ethanol; DMSO, dimethylsulfoxide; INDO, indomethacin; NDGA, nordihydroguaretic acid.

Solutions were prepared as described in Methods. AA (30 μM) was exposed to 0.1 ppm for 30 min. Concentrations stated are final concentrations. In some instances, PBS and O₃-exposed AA (O₃-AA) solutions were pretreated with CAT (120 U/ml), SOD (107 U/ml), and EtOH (1 mM) for 1 hr at 37°C before addition to PMN. In some experiments, PMN (in PBS) were preincubated with INDO and NDGA in DMSO or DMSO alone (15 min, 37°C, with rocking) before incubation with test solutions.

*Significant difference from PBS-incubated cells, *p* < 0.05

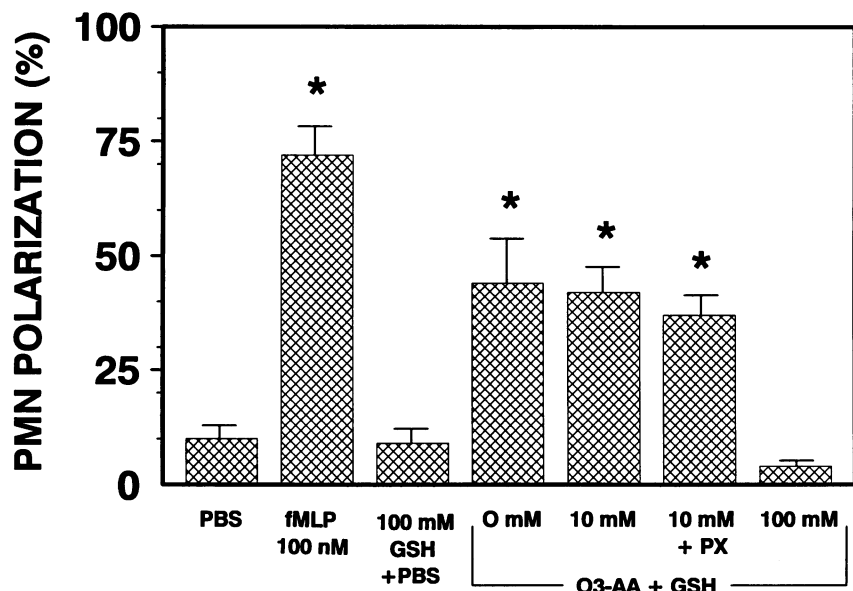


Figure 5. Effect of glutathione (GSH) on O_3 -exposed arachidonic acid (AA) induction of polymorphonuclear leukocyte (PMN) polarization. We pretreated 900 μ l of O_3 -exposed AA (0.1 ppm for 30 min) with 0, 10, or 100 mM GSH or 10 mM GSH and GSH peroxidase (Px; 5 U/ml) in 0.1 ml (final volume 1.0 ml) for 60 min at 37°C. As an additional control, PBS was also pretreated with 100 mM GSH. We then added 100 μ l of each solution to PMN and assessed polarization. Significant difference from PBS alone, $p < 0.05$.

was significantly greater than the production of O_2^- by PBS-incubated control cultures (2.6 ± 1.8 nmol; $n = 4$, $p < 0.05$). PMN incubated for 60 min with air-exposed AA (30 min), O_3 -exposed PBS (0.1 ppm for 30 min), or O_3 -exposed AA (0.1 ppm for 30 min) produced 2.3 ± 0.6 , 2.6 ± 1.4 , and 3.5 ± 1.0 nmol O_2^- , respectively, which was similar to the amount produced by PBS-incubated control cultures. The effect of pretreatment of PMN with O_3 -exposed AA (0.1 ppm for 30 min) on a subsequent stimulation of O_2^- production by PMA was also examined. There was no difference in the PMA-stimulated production of O_2^- between cells pretreated (15 min, 37°C) with O_3 -exposed AA and cells pretreated with vehicle (data not shown).

T-lymphocyte cultures that were preincubated for 60 min with air-exposed PBS or AA (120 min) and then subsequently incubated with either PHA or concanavalin A had a similar proliferation, as assessed by 3H -thymidine incorporation, as control cultures incubated with PBS (data not shown; $p = NS$). Lymphocytes incubated with PBS that had been exposed to 0.1 ppm or 1.0 ppm O_3 for 15–120 min had a similar proliferation as cultures incubated with unexposed PBS (Table 4). In contrast, 1.0 ppm O_3 -exposed AA (for 120 min) decreased lymphocyte PHA-stimulated and concanavalin A-stimulated proliferation to 56% and 27% of PBS control values, respectively ($p < 0.05$). AA exposed to 0.1 ppm O_3 for 15 min inhibited concanavalin A-stimulated lymphocyte mitogenesis to 76% of control but did not alter

PHA-stimulated mitogenesis. In separate experiments, the effect of H_2O_2 and O_2^- on decreased lymphocyte proliferation induced by 1.0 ppm O_3 -exposed AA (120 min) was examined. PHA-stimulated lymphocyte cultures incubated with O_3 -exposed AA and catalase and SOD pretreated O_3 -exposed AA had $17 \pm 12\%$ and $12 \pm 8\%$ proliferation, respectively, compared to control cultures. Similarly, concanavalin A stimulated T-cells incubated with untreated and catalase- and SOD-treated O_3 -exposed AA had $5 \pm 3\%$ and $5 \pm 3\%$ of the control proliferation value, respectively. Cultures incubated with catalase-treated and SOD-treated O_3 -exposed PBS (1.0 ppm for 120 min) had similar proliferation as controls (data not shown).

NK cells were pretreated for 60 min with test solutions and then incubated with

^{51}Cr -labeled K562 target cells. O_3 -exposed AA (either 1.0 ppm for 120 min or 0.1 ppm for 30 min) significantly decreased NK cytotoxicity to $20 \pm 10\%$ and $24 \pm 5\%$ of PBS-incubated control cultures ($p < 0.05$; Table 5). NK cells incubated with PBS exposed to O_3 (0.1 ppm for 30 min or 1.0 ppm for 120 min) did not change NK killing of target cells from control cultures. In a separate set of experiments, O_3 -exposed AA that was preincubated with catalase and SOD before incubation with NK cells slightly, but nonsignificantly, attenuated the decrease in NK cytotoxicity. Untreated O_3 -exposed AA (0.1 ppm for 30 min) had $13.7 \pm 8.4\%$ of control NK activity, whereas catalase-treated and SOD-treated O_3 -exposed AA had $32.6 \pm 11.9\%$ of control NK cytotoxicity ($p = NS$).

The effect of O_3 -exposed AA on the binding of NK cells to target cells was then examined as a possible mechanism for the decreased NK cytotoxicity of target cells. In a separate set of experiments, NK cells were incubated with PBS, O_3 -exposed PBS (0.1 ppm for 30 min), or O_3 -degraded AA (0.1 ppm for 30 min), and binding to K562 cells was assessed. There was no significant change in the percentage of effector cells binding to target cells, as PBS-incubated NK cells formed $10.4 \pm 1.0\%$ conjugates, O_3 -exposed PBS-incubated cells had $11.5 \pm 2.0\%$ conjugates, and O_3 -exposed AA incubated cells had $9.8 \pm 3.1\%$ conjugates ($n = 3$, $p = NS$).

Discussion

There are several potential sources of lung AA that can be attacked by exposure to O_3 . Free AA can exist in the lung lining fluid after release from lung cells (e.g., alveolar macrophages or tracheal epithelial cells) (38,39). A second source of free AA in the lung is the O_3 -induced increase in free extracellular AA, possibly due to the stimulation of various phospholipases (40) and the inhibition of AA reacylation that

Table 4. Effect of O_3 -exposed arachidonic acid (AA) on human peripheral blood lymphocyte proliferation

Mitogen	O_3 (ppm)	Exposure time (min)	Solution	Proliferation (% control)
Concanavalin A	1.0	120	PBS	85 ± 10
	0.1	15	AA	$27 \pm 3^*$
Phytohemagglutinin	1.0	120	PBS	104 ± 4
	0.1	15	AA	$76 \pm 10^*$
	1.0	120	PBS	95 ± 7
	0.1	15	AA	$56 \pm 7^*$
			PBS	114 ± 1
			AA	99 ± 7

PBS, phosphate-buffered saline. Solutions were exposed to O_3 (0.1 or 1.0 ppm) for either 15 or 120 min. Lymphocytes were incubated with the solutions (60 min), and cells were subsequently cultured for 72 hr in the presence of mitogen. Proliferation was then assessed as the amount of 3H -thymidine incorporated by the cultures. Proliferation was compared to cells incubated with unexposed PBS. Concanavalin A-stimulated and phytohemagglutinin-stimulated control cultures incorporated $1.29 \pm 0.09 \times 10^5$ dpm and $1.34 \pm 0.10 \times 10^5$ dpm, respectively ($n = 4$ for all values).

*Significant difference from PBS control culture, $p < 0.05$

Table 5. Effect of O₃-exposed AA on human peripheral blood natural killer (NK) cell lysis of K562 tumor cells

Solution	O ₃ (ppm)	Exposure time (min)	NK lysis (% control)	n
PBS	0.1	30	108 ± 11	15
AA	0.1	30	24 ± 5*	15
PBS	1.0	120	93 ± 8	10
AA	1.0	120	20 ± 10*	10

PBS, phosphate-buffered saline. Solutions were exposed to O₃ (0.1 or 1.0 ppm) for either 30 or 120 min. Peripheral blood NK cells were incubated with the solutions for 60 min, and cells were subsequently incubated with ⁵¹Cr prelabeled K562 cells at different effector:target ratios (50:1 to 1.8:1) for 4 hr at 37°C. Release of ⁵¹Cr into the media was measured and lysis expressed as LU₁₀. PBS-incubated control cultures averaged 35 ± 9 activity units (n = 17).

*Significant difference from PBS-incubated control cells, p < 0.05.

has been demonstrated with H₂O₂ (41). Increased concentrations of AA have been found in the lung lavages of rats exposed to 1.1 ppm O₃ *in vivo* (42). Exposure of rat and human alveolar macrophages (12, 43,44), bovine tracheal epithelial explants (45), and BEAS-S6 cells (46) to O₃ *in vitro* results in an increased release of AA or AA metabolites, suggesting an increased release of free AA.

In this study, we exposed AA in a cell-free aqueous environment to O₃ as an *in vitro* model for the reaction of this polyunsaturated fatty acid with O₃. We have demonstrated that AA can be degraded by O₃ in a cell-free system at concentrations as low as 0.1 ppm. The major products of this degradation are aldehydic compounds (as determined by reaction with DNPH and separation by HPLC; Fig. 1) and H₂O₂ (Table 1), with only a small amount of other peroxides formed. More H₂O₂ was formed with the longer O₃ exposure period, but less aldehydic substances were formed. The formation of similar products, i.e., carbonyl compounds and hydrogen peroxide (but only small amounts of hydroperoxides), upon O₃ exposure of oleic acid (2.5 mM) emulsions and dioleoyl phosphatidylcholine liposomes (2 mM) in aqueous media has been previously reported (14,15). O₃ can also degrade some AA metabolites in cell-free systems into more polar products (12,46), presumably through a mechanism similar to AA degradation, resulting in formation of carbonyl products and H₂O₂. Similarly, other polyunsaturated fatty acids may be degraded by O₃ exposure. Polyunsaturated fatty acids have been reported to have high reactivity with O₃ relative to other biological molecules (47), suggesting that O₃ can also react relatively well with these fatty acids in the lung. Exposure of rodents to O₃ *in vivo* has been reported to

degrade AA in the lung (13). The precise nature of the AA degradation products in the rodent lungs were not elucidated because the degradation products (postulated to include carbonyl compounds and ozonides) were converted to carboxylic acids for analysis. It was not known which lung source of AA, i.e., intracellular and/or extracellular, was degraded by O₃.

Another source of lung AA that may be attacked by O₃ is AA that is esterified in phospholipids and neutral lipids in lung fluid and cells. AA composes 0.4–7.5% (by weight) of various phospholipids (including phosphatidylcholine) recovered by lavage of human lungs (11). In bronchoalveolar lavage fluid recovered from healthy human subjects, the concentration of AA (both free and esterified) is approximately 318 nM (G. Hatch, personal communication; 48). After correction for a dilution factor of 100 in the bronchoalveolar lavage fluid (due to the instillation of saline to wash out the lining fluid) based on the urea concentration technique (2), it is estimated that the concentration of AA would be approximately 31.8 μM in epithelial lining fluid. Therefore, we believe that the AA concentrations used in these present studies are relevant to physiological levels *in vivo*. Furthermore, AA attached at the C-2 position in phosphatidylcholine has been shown to be degraded to an aldehyde upon exposure *in vitro* to O₃ (49). Additionally, increased release of arachidonyl-containing phospholipids and neutral lipids from cells has been reported in rat alveolar macrophages exposed *in vitro* to 0.1–1.0 ppm O₃ (12), suggesting that esterified AA may be degraded by O₃ outside the cells.

Once formed, the persistence of AA-derived aldehydic substances in the lung lining fluid would depend on the chemical stability of the aldehydes, the interaction of the aldehydes with catabolic substances, uptake into cells, and transport from the lung. The O₃-exposed AA products had an innate stability, demonstrated by the fact that <16% of each peak was degraded at room temperature after 24 hr in the dark. At least some of the aldehydic substances react with GSH (Fig. 2), although 100 mM GSH is required to react with all of the less polar aldehydes (peaks III–V in Fig. 2). Concentrations of GSH in human epithelial lining fluid from healthy subjects have been reported to be <0.5 mM (50) using urea as a reference compound. These data imply that GSH alone would not be a likely detoxifying compound for aldehydes derived from AA once the carbonyls have been formed. However, GSH in conjunction with GSH transferase has been shown to catabolize aldehydes (51) and may enhance the degradation of O₃-

exposed AA. Additionally, GSH may modulate the formation of these aldehydic compounds through scavenging O₃ directly, as sulfhydryl-containing groups can react readily with O₃ (47). GSH peroxidase (in the presence of 10 mM GSH) did not alter the size of peaks III–V (Fig. 2), indicating these products are not peroxides and confirming the results of the MCDP assay that there are few lipid peroxides present in O₃-degraded AA.

Besides the possible reaction of O₃ with polyunsaturated fatty acids in the lung lining fluid, O₃ may directly react with lung cells. O₃ exposure *in vivo* has been shown to induce biochemical changes in human alveolar macrophages recovered by bronchoalveolar lavage (52) and oxygen-18, derived from ¹⁸O-labeled O₃, has been reported to reach alveolar macrophages *in vivo* (53), suggesting that O₃ can reach distal portions of the lung. Airway epithelial cells are likely to be exposed to O₃ (and/or to the O₃-induced degradation products formed in the airway lining fluid) due to the proximal lung location. We used a human bronchial epithelial cell line (BEAS) as a model of airway epithelial cell exposure to O₃. BEAS cells exposed *in vitro* to 0.1 ppm and 1.0 ppm O₃ for 60 min released aldehydic substances derived from incorporated ³H-AA (Fig. 3). Some of these aldehydic AA products derived from the BEAS-S6 cells eluted with retention times similar to AA degraded by O₃ in a cell-free system, suggesting that the products formed in the cell-free model system have relevance to the study of aldehydic substances formed by biological systems in response to O₃. There were also some aldehydic AA products formed upon exposure of BEAS cells to O₃ that did not elute with similar retention times as AA degraded in the cell-free system. This may be due to different kinetics of formation of aldehydic products by the cells, and/or the derivation of aldehydic substances from cellular phospholipids present in the exposures of BEAS cells (but not free AA) to O₃. The production of only low levels of H₂O₂ by O₃-exposed BEAS cells suggests that either the actual mass of degraded AA was too low to detect an increase in H₂O₂ and/or the BEAS cells degraded H₂O₂ very quickly.

The exact dosimetry between the O₃ reaching bronchial epithelial cells upon *in vivo* exposure and in our *in vitro* exposure system using BEAS cells is not known. However, there are two lines of evidence that suggest that the *in vitro* exposures have relevance to *in vivo* studies. First, there was a transient, concentration-dependent (0.1–1.0 ppm) decrease in BEAS cell viability (measured by the release of chromium-51) upon exposure to O₃ (54),

which may account in part for some of the production of aldehydic substances. Similarly, monkeys exposed to 0.5–0.8 ppm O_3 *in vivo* (8 hr/day for 7 days) had a loss of ciliated cells in the conducting airways with subsequent hypertrophy and hyperplasia of the remaining cells (55,56). Secondly, we have reported that this cell type produces a concentration-related increase in eicosanoids (e.g., thromboxane B_2 and prostaglandin E_2) and cytokines (e.g., IL-6) upon exposure to O_3 (0.1–1.0 ppm for 1 hr) (57), similar to the increased amounts of these eicosanoids and cytokines found in the bronchoalveolar lavage fluid recovered from human subjects exposed to 0.1–0.6 ppm O_3 for 2.0–6.6 hr (1–3).

In the present study, we examined the biological activities of the O_3 -exposed AA using several different assay systems. As previously discussed, a PMN influx into the lungs of O_3 -exposed humans and rodents is a hallmark of O_3 toxicity. O_3 -exposed AA (0.1 ppm for 30 min) induced an increased polarization in human PMN (Table 2). The O_3 concentration and exposure duration are important in the increased polarization, as a 1.0-ppm exposure for 120 min did not cause an increased PMN polarization. This lack of polarization in the 1.0 ppm O_3 exposure for 120 min correlates with a different profile of aldehydic AA products formed compared to the 0.1 ppm exposure (Fig. 1). The change in PMN shape induced by the O_3 -exposed AA (0.1 ppm for 30 min) was confirmed by a decrease in the mean channel forward-angle light scatter of the population, similar to decreases observed using another chemoattractant agent, i.e., fMLP (Fig. 4), and reported by other investigators (32).

The possible reactants in the solution of O_3 -exposed AA (0.1 ppm for 30 min) were then examined for their contribution to the increase in PMN polarization (Table 3; Fig. 5). Pretreatment of the O_3 -AA with catalase, SOD, and ethanol before incubation with PMN did not attenuate the increased polarization, nor did H_2O_2 (3 μM) increase polarization. These data suggest that H_2O_2 and O_2^- and hydroxyl radical were not important in this PMN response. The increased PMN polarization induced by the 0.1 ppm O_3 -exposed AA also did not appear to be due to any residual AA present for the following reasons: 1) 3 nM AA (equivalent to the maximal concentration of residual AA observed in our HPLC chromatograms) did not increase PMN polarization; 2) PMN required cyclooxygenase and/or lipoxygenase activity to polarize in response to 3 μM AA, as indomethacin and NDGA pretreatment decreased AA-induced polarization; 3) in contrast to pure AA, O_3 -AA appears

to act directly on PMN to induce polarization without metabolism by cyclooxygenase and/or lipoxygenase, as indomethacin and NDGA did not alter the increased polarization; 4) pretreatment of O_3 -AA with 100 mM GSH (which eliminates most of peaks III–V; Fig. 2), prevented the increase in PMN polarization. These data suggest that the aldehydic products (peaks III–V) are responsible for the PMN polarization.

The effect of O_3 -exposed AA (0.1 ppm for 30 min) on human PMN responses appeared to be specific. O_3 -exposed AA increased polarization, but did not increase the production of O_2^- , either unstimulated or in response to PMA. The effects of O_3 exposure on human PMN O_2^- production are not known. However, cells recovered by bronchoalveolar lavage from human subjects exposed to 0.4 ppm O_3 for 2 hr (primarily macrophages but with approximately 10% PMN) had no change in spontaneous or stimulated O_2^- production (2,3). Acrolein (5–40 mM), but not propanal, has been reported to decrease human and rat PMN spontaneous and PMA-stimulated O_2^- production (58), suggesting that the aldehydic substances of O_3 -exposed AA either are not structurally like acrolein (i.e., not α,β -unsaturated aldehydes like acrolein), or are not produced in high enough concentrations to inhibit PMN O_2^- generation.

Human peripheral blood T-cells have been shown to have a decreased mitogen-stimulated proliferative response upon exposure to O_3 *in vivo* (4–6). We have demonstrated that O_3 -exposed AA (1.0 ppm for 120 min) decreased mitogenesis in response to PHA and concanavalin A (Table 4). This decrease in T-cell proliferation was not due to decreased lymphocyte viability. AA exposed to less O_3 (i.e., 0.1 ppm for 30 min) induced a significant decrease in concanavalin A-stimulated proliferation, but not in PHA-stimulated mitogenesis. This differential effect of 0.1 ppm O_3 -exposed AA on concanavalin A-stimulated proliferation may be due to a decreased function of monocytes (which are needed as an accessory cell to a greater extent in concanavalin A-induced T-cell proliferation compared to PHA-induced proliferation) upon exposure to O_3 -exposed AA (7). These data regarding T-cell mitogenesis are similar to the PMN polarization data in that the profile of AA degradation products formed (Fig. 1) affects the magnitude of biological response. Neither H_2O_2 nor O_2^- appear to play a role in the inhibition of lymphocyte proliferation induced by O_3 -exposed AA (1.0 ppm for 120), as pretreatment with catalase and SOD did not attenuate the decreased mitogenesis. These data suggest that the

aldehydic substances are responsible for the decreased T-cell response. In support of this, 5-nitro-2-nitrofuralddehyde (at <35 μM) has been shown to almost totally inhibit human blood T-cell proliferation (59).

Both 0.1 ppm (for 30 min) and 1.0 ppm (for 120 min) O_3 -exposed AA decreased human peripheral blood NK lysis of K562 tumor target cells to a similar extent (Table 5). There was no decrease in NK viability upon incubation with O_3 -exposed AA, implying that an alteration in NK cell viability was not the mechanism responsible for decreased target cell lysis. H_2O_2 and/or O_2^- may be partially involved in the decreased lysis of target cells, as pretreatment of the O_3 -exposed AA (0.1 ppm for 30 min) with catalase and SOD only slightly (approximately 20%), but nonsignificantly, attenuated the decreased cytotoxicity. H_2O_2 has been shown to decrease NK activity toward K562 target cells (20). The mechanism involved in the decreased NK cytotoxicity toward K562 cells appeared to involve a step in the lytic portion of NK cytotoxicity and not the binding phase because O_3 -exposed AA had no effect on the attachment of the effector cells to the target cells.

In summary, we have shown that AA can be almost completely degraded by O_3 in an aqueous environment at concentrations as low as 0.1 ppm for 30 min. The major products formed by this degradation process are primarily aldehydic substances and H_2O_2 . Similar aldehydic products are formed when bronchial epithelial cells are exposed to O_3 *in vitro*. These aldehydic products possess bioactivity as evidenced by increased PMN polarization, decreased peripheral blood T-cell mitogenesis, and decreased NK cytotoxicity, which may play a role in the responses to O_3 exposure. The presence of these degradation products in the lung and the association of the O_3 -exposed AA with altered lung function remain to be determined.

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