Interfacial phospholipids inhibit ozone-reactive absorption-mediated cytotoxicity in vitro

Lydia M. Connor, Carol A. Ballinger, Thomas B. Albrecht, and Edward M. Postlethwait

Interfacial phospholipids inhibit ozone-reactive absorption-mediated cytotoxicity in vitro. Am J Physiol Lung Cell Mol Physiol 286: L1169–L1178, 2004. First published January 16, 2004; 10.1152/ajplung.00397.2003.—The intrapulmonary distribution of inhaled ozone (O3) and induction of site-specific cell injury are related to complex interactions among airflow patterns, local gas-phase concentrations, and the rates of O3 flux into, and reaction and diffusion within, the epithelial lining fluid (ELF). Recent studies demonstrated that interfacial phospholipid films appreciably inhibited NO2 absorption. Because surface-active phospholipids are present on alveolar and airway interfaces, we investigated the effects of interfacial films on O3-reactive absorption and acute cell injury. Compressed films of dipalmitoyl-glycero-3-phosphocholine (DPPC) and rat lung lavage lipids significantly reduced O3-reactive absorption by ascorbic acid, reduced glutathione, and uric acid. Conversely, unsaturated phosphatidylcholine films did not inhibit NO2 absorption. We evaluated O3-mediated cell injury using a human lung fibroblast cell culture system, an intermittent tilting exposure regimen to produce a thin covering layer, and nuclear fluorochrome permeability. Exposure produced negligible injury in cells covered with MEM. However, addition of AH2 produced appreciable (<50%) cell injury. Film spreading of DPPC monolayers necessitated the use of untilted regimens. Induction of acute cell injury in untilted cultures required both AH2 plus high O3 concentrations. Addition of DPPC films significantly reduced cell injury. We conclude that acute cell injury likely results from O3 reaction with ELF substrates.

Address for reprint requests and other correspondence: E. M. Postlethwait, Dept. of Environmental Health Sciences, School of Public Health, Univ. of Alabama at Birmingham, RPHB 530, 1530 3rd Ave. South, Birmingham, AL 35294-0022 (E-mail: epostlethwait@ms.sphp.uab.edu).

http://www.ajplung.org 1040-0605/04 $5.00 Copyright © 2004 the American Physiological Society

OZONE (O3) IS THE PRINCIPAL oxidant component of photochemical smog and is among the most extensively occurring tropospheric toxicants. The effects of both acute and chronic exposure to O3 have been well characterized (12, 24) and are of particular concern in preexisting lung diseases, in children, and in the elderly (13, 38, 42). The distribution of epithelial injury resulting from acute exposure is likely related to the intrapulmonary gas-phase dispersion of O3 and its local rate of absorption into the air space surface fluid compartment [epithelial lining fluid (ELF)]. O3 absorption in the lung is, in part, governed by the rate at which O3 dissolves in the ELF and the chemical reactions with ELF constituents such as surfactant, epithelial lining fluid; interfacial resistance; antioxidants that ensue (4, 22, 23, 30). This process, termed “reactive absorption” (4, 23), is thought to occur within a reaction zone near the gas-liquid interface. With the use of O3 aqueous diffusion coefficients and bimolecular reaction rate constants, conservative estimations of the extent of O3 penetration into the ELF (axial penetration) of human airways suggest a reaction zone thickness of ~0.1 μm (19, 31, 32).

Because facile reactions within the biochemically complex ELF likely constrain O3 diffusion, it has been assumed that specific product(s) of reactive absorption initiates the cascade of cytotoxic events that lead to cell injury (31, 34). Consequently, understanding the processes that influence local flux rates is critical to elucidating both the mechanisms of O3-induced cell injury and dose-response relationships. Pulmonary surfactant, a composite mixture of glycerophospholipids, cholesterol, and surfactant-associated proteins, functions to prevent alveolar collapse at end-expiratory lung volumes (9). Although the interfacial monolayer of surface-active phospholipids is assumed to impart limited resistance to the flux of respiratory gases, recent in vitro studies demonstrated that compressed interfacial films of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), the principal surface-active component of pulmonary surfactant, introduced appreciable resistance to the flux of nitrogen dioxide (NO2) from the gas phase into the aqueous phase (10). DPPC significantly reduced NO2 uptake, irrespective of aqueous substrate concentration, composition, or NO2 gas-phase concentration. Furthermore, neither reducing aqueous-phase surface tension (γ) with detergents nor compression of unsaturated phospholipid films altered the rates of NO2-reactive absorption, suggesting that the resistance imparted by the DPPC films was directly attributable to the presence and physical properties of the interfacial saturated phospholipids.

O3 and NO2 are only modestly soluble in aqueous solutions but undergo comparably facile reactions with a variety of dissolved biomolecules. However, the two toxicants display notable toxicity differences (O3 ~10–15 times greater than NO2), differentially react with monounsaturated fatty acids, and have somewhat different structural configurations (12). In considering the penetration of gases through interfacial films, both the molecular packing of film molecules (compression) and the effective cross-sectional area of the permeating molecular species may be critical. Relatively minor changes in fatty acid composition (e.g., substitution of the β-position palmitic acid with oleic acid) attenuate the film resistive properties

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
toward NO2 flux (10). Similarly, variations in the physical characteristics of the permeating gas may influence its flux through the resistive barrier of the gas-liquid interface. It is, therefore, possible that the three-dimensional structural differences between NO2 (134° bond angle, 1.19 Å bond length) and O3 (117° bond angle, 1.28 Å bond length) and dissimilar fatty acid reactions might influence film permeation.

Because interfacial films are present in both the alveolar spaces and conducting airways (9, 15, 18, 25, 35, 40), factors that govern the rate of O3 interfacial transfer in conjunction with its ELF reaction and diffusion may influence product formation, the specific cytotoxic products that diffuse to the underlying epithelium, and thus the mechanisms and dose-effect relationships of exposure-induced cellular responses. Consequently, we investigated the contribution of interfacial films to O3-induced cytotoxicity under conditions that mimicked the compartmentation of the respiratory tract surfaces. To accomplish this, we examined the effects of interfacial phospholipid films on O3 absorption in vitro with subsequent studies focused on the combined influences of interfacial films and extracellular chemistry on exposure-related acute cell injury.

MATERIALS AND METHODS

DPPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG), and 1,2-dilinoleoyl-sn-glycero-3-phospholipid (DLPC), all in powder form, were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. GSH, ascorbic acid (AH2), uric acid (UA), desferrioxamine (DFX), SDS, Dulbecco’s PBS with calcium and magnesium, Eagle’s MEM, FBS, trypsin, and all other cell culture media and reagents were purchased from Sigma Chemical (St. Louis, MO). The fluorescent dyes, 4,6-diamidino-2-phenylindole (DAPI) and ethidium homodimer-1 (EthD-1), were purchased from Molecular Probes (Eugene, OR). HPLC grade chloroform, methanol, propanol, and ethidium homodimer and all other reagents of analytic grade were purchased from Fisher Scientific (Houston, TX).

Surfactant Isolation

Surfactant was isolated from viral antigen-free, 250- to 274-g male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). The protocol for harvesting bronchoalveolar lavage fluid (BALF) was approved by the Animal Care Use Committee. The surface-active components of BALF were harvested as described previously (10), with minor modifications. Briefly, after induction of anesthesia and tracheal cannulation, a midline thoracotomy was performed, and lungs were lavaged in situ with 10 ml of 0.15 M NaCl and subsequently two additional times with 8.0 ml. The BALF was pooled, centrifuged at 150 average g for 10 min to remove cells and debris, and further centrifuged at 60,000 average g for 2.5 h to isolate the surface-active components. The supernatant was discarded, the pellet resuspended in PBS, and the lipids extracted by a modified method of Bligh and Dyer (5) with double extraction of the upper phase. A 1 mg/ml solution in chloroform was prepared based on gravimetric estimation of total lipid. The extract was stored under N2 at −20°C and used in probe exposures (see below) within 24 h.

Monolayer Compression Studies

The exposure probe-surface balance (Fig. 1) utilized to evaluate O3 absorption through phospholipid monolayers has been described in detail previously (10). Films were deposited from chloroform solutions onto an aqueous surface confined within a Teflon-lined parallelogram compression frame (total surface area = 357 cm2). Just before deposition, particles on the aqueous surface were removed by aspiration. The chloroform vehicle evaporated within a few seconds of initial deposition. The compression frame was mounted in a Plexiglas dish, which contained the aqueous bulk phase (500 ml), and the interfaces were compressed by using a manual crank shaft. Interfacial surface areas were reduced to a maximum of 60%; further compression was precluded due to the presence of the exposure probe inside the compression frame. The exposure probe (see below), which delivered O3 over a defined surface area, was mounted on a movable stand and was positioned to a distance of 1 mm from the probe edge to the aqueous surface (10).

O3 Exposures of Pure Chemical Systems

All pure chemical exposures were performed under quasi-steady-state, gas-phase conditions at room temperature (25°C) with the aqueous phase unsterilized. Solutions of AH2, GSH, or UA were utilized as aqueous substrates for O3 absorption. DFX (50 μM) was added to AH2 solutions to limit Fe-initiated autooxidation. Exposures were conducted utilizing either the probe and parallelogram compression apparatus or a flask exposure vessel, wherein small glass Erlenmeyer flasks fitted with Teflon stoppers and inlet and exit ports were employed (23). Flask exposures were conducted when it became necessary either to limit the aqueous phase volume or to prevent contamination of the compression system (e.g., detergent studies). Total flow of pollutant gas through both exposure systems exceeded the rate of gas-phase sampling by 10–15%. O3 was produced by passing 100% O3 through a silent arc electrode (model 25, Sander Elektroapparat, Uetze-Eltze, Germany), a portion of which was added by countercurrent injection of O3 into temperature-equilibrated, humidified air by using mass flow controllers (Scott Specialty Gas, Houston, TX). Adjusted to achieve the appropriate final O3 concentrations, O3 gas-phase concentrations were continuously monitored (model 49 Analyzer, Thermoenvironmental, Franklin, MA). O3 absorption was computed based on the mass balance across the exposure system and expressed as either J) the absorption rate per unit surface area [(O3)inlet − (O3)exit]/sample flow-surface area⁻¹], where (O3)inlet is inlet O3 concentration and (O3)exit is exit O3 concentration.
or 2) the fractional uptake (absorbed dose/delivered dose) (23). Absorption data were normalized to fractional uptakes to compensate for dose rate variations imposed by modest deviations in flow rates and/or the gas-phase \( \text{O}_3 \) concentrations.

For compression system exposures, 500-ml buffer (10 mM phosphate, pH 7.0), with or without \( \text{AH}_2 \) (25–250 \( \mu \text{M} \)), GSH (1.0 mM), or UA (250 \( \mu \text{M} \), were placed in the Plexiglas dish, and the aqueous surface was cleaned by aspiration. The system was raised to achieve a 1-mm separation between the bottom edge of the probe and the aqueous surface. The probe gas inlet and exit lines were connected, and the system enclosure was sealed. After a 10-min control exposure period, the phospholipid monolayer was deposited by using a 25-\( \mu \text{l} \) Hamilton syringe to achieve an initial surface density of 121 \( \text{A}^2 /\text{molecule} \) (0.1 \( \mu \text{g/cm}^2 \)) and allowed to equilibrate for 5 min. Subsequently, three compression-expansion cycles were performed before compression to the final set point(s). All exposures generally lasted 15–20 min. The initial \( \text{O}_3 \) concentration was determined before and subsequent to each exposure with the probe or flask out of line.

\( \gamma \) Measurements

The \( \gamma \) was measured by using a flamed 2.5-cm-wide roughened platinum plate. Before phospholipid deposition, the aqueous surface was thoroughly cleaned by aspiration such that \( \gamma = 71 \pm 3 \text{ mN/m} \) was maintained during several compression-expansion cycles. The \( \gamma \) was measured directly on all monolayers studied in the probe and parallelogram compression apparatus. However, due to the inability to perform plate measurements within the exposure flasks, \( \gamma \) measurements for the flask studies were determined on representative films contained in open-glass vessels.

The \( \gamma \) was also measured on representative cell culture systems at room temperature because the surface balance could not be installed within an incubator. Immediately before \( \gamma \) measurements, the media were aspirated, dishes were washed twice with PBS, and 3.0 ml of MEM (exposure media) were added to the dish. To accommodate the use of the platinum plate, the volume of media applied exceeded that used in exposure studies. The \( \gamma \) were measured before (5 min) and after (30 min) DPPC deposition (3.5 \( \mu \text{l} \) of 4 mg/ml stock solution; 0.5 \( \mu \text{g DPPC/cm}^2 \) final surface density).

Cell Culture Model

We utilized a human embryonic lung fibroblast cell line (LU cells), originally established in the late 1970s (T. A. Albrecht (1, 6)) for all cell culture studies. These cells, which served expressly as targets to assess acute toxicity, were specifically selected due to their 1) oxidant sensitivity (see results), 2) minimal secretory activity, and 3) relatively minor effects on media concentrations of added \( \text{AH}_2 \) during 1-h air exposures. The LU cells were maintained in 162-cm\(^2\) tissue culture flasks, as described previously (1, 6), utilizing Eagle’s MEM plus 10% FBS, 0.022% sodium bicarbonate, 2 mM glutamine, 100 U/ml of penicillin, and 100 \( \mu \text{g/ml streptomycin} \) (growth medium). Cells were harvested with trypsin, and log growth cells were obtained by seeding LU cells at a density of 1.5 \( \times \) \( 10^4 \) cells/cm\(^2\) in sterile 60-mm tissue culture dishes and cultivating 18–20 h (achieving 35–50% confluency) in growth medium. Log growth cells were employed due to their enhanced oxidant sensitivity.

Hydrogen Peroxide Exposures

As a positive control for oxidant insult, LU cells were exposed to 500 \( \mu \text{M} \text{H}_2\text{O}_2 \) (initial concentration) for 60 min at 37°C under untilted conditions in a tissue culture incubator (see below). \( \text{H}_2\text{O}_2 \) solutions were prepared immediately before use. Conditioned growth medium was collected and pooled, and 0.1 ml/cm\(^2\) of MEM or MEM-contaminating \( \text{H}_2\text{O}_2 \) was added to each dish. To end the exposure, the exposure media were aspirated, and pooled conditioned medium was replaced. Cells were incubated for 1 h postexposure and stained as described below.

\( \text{O}_3 \) Exposure of Cell Cultures

Only acute cell injury studies were performed to limit exposure duration effects on film stability and to maintain control of extracellular (media) conditions. As described above, immediately before exposure, growth medium was removed, pooled in sterile tubes, and stored at 37°C for use postexposure as conditioned medium. Adherent cells were washed twice with PBS followed by the addition of phenol red-free MEM (exposure media), with or without 500 \( \mu \text{M} \text{AH}_2 \). \( \text{AH}_2 \) served as the absorption substrate based on its facile reaction with \( \text{O}_3 \) (22, 28) and preliminary studies demonstrating that the \( \text{O}_3 + \text{AH}_2 \) reaction yields product(s) that initiates secondary oxidative events. For all cell culture exposures, 50 \( \mu \text{M} \text{DFX} \) were added to the MEM to control for any potential DFX + \( \text{O}_3 \) interactions and/or cellular effects from iron chelation; no other additional solutes (e.g., serum) were added, except where noted.

Cell exposures were conducted in a custom-designed exposure system (E. M. Postlethwait) that included specially fabricated glass exposure chambers housed in modified tissue culture incubators to maintain constant temperatures within the exposure chambers (control and \( \text{O}_3 \)). Gases flowed in through a single port in the chamber \( \text{O}_3 \) and out through two diagonally located ports in the lower chamber walls. Culture dishes were mounted on a perforated stainless steel support platform and held in place by small posts that protruded through the platform perforations. The chambers were equipped with a specially designed “baffle” that was annealed to the inside of the chamber lid through which the inlet gases flowed. The baffle introduced a uniform dispersion of \( \text{O}_3 \) throughout the chamber. This was verified by measuring \( \text{O}_3 \) absorption among glass petri dishes spaced across the support platform. Absorption was determined by comparing the post-exposure optical density of potassium iodide solutions (20) within the petri dishes. Ninety-five percent air-5% \( \text{CO}_2 \) flowed through a temperature-controlled bubbler (42°C) located inside the incubator and filled with sterile water, which produced humidified atmospheres (>97% relative humidity) within the chambers. High-concentration \( \text{O}_3 \) generated via a silent arc electrode (from 100% \( \text{O}_3 \)), equipped with a line conditioner, voltage stabilizer, and a variable transformer, was diluted into the flow of air-\( \text{CO}_2 \) via countercurrent injection downstream of the humidifier. All gas flows were regulated via mass flow controllers with total gas flow through the chambers of \( \sim 2.100 \text{ml/min} \) (≈1 air change/2 min). Exposure atmospheres [1–5 parts/ million (ppm) \( \text{O}_3 \)] were quantified as above. Perma Pure dryer (Toms River, NJ), which removed water vapor via a countercurrent flow of dry \( \text{N}_2 \) and was unreactive toward \( \text{O}_3 \), was placed in line between the chamber sample port and the \( \text{O}_3 \) analyzer to prevent moisture accumulation and limit the effects of water vapor on analyzer accuracy. The \( \text{O}_3 \) chamber was conditioned for at least 1 h before initiation of exposures.

Two exposure protocols were utilized: 1) either exposure chambers were cyclically tilted (2-min hold time per tilt) to intermittently produce a thin aqueous film over the upper one-half of the cell culture dishes, or 2) the chambers remained untilted. Tilting experiments were initially utilized to determine the exposure and media (aqueous substrate) conditions necessary to induce acute cell injury. For these studies, 0.1 ml/cm\(^2\) MEM-50 \( \mu \text{M} \text{DFX} \) (±500 \( \mu \text{M} \text{AH}_2 \)) was added to cover cells. In unilted experiments, which evaluated the DPPC monolayer-induced effects on \( \text{O}_3 \)-mediated acute cell injury, the volume of media covering cells was 0.048 ml/cm\(^2\). DPPC films were deposited onto minimal aqueous phases after dishes were placed in the exposure chambers. Care was taken to ensure that the stainless steel support platforms were level to facilitate even media distribution over the cells. Three and one-half microliters of DPPC stock solutions (4 mg/ml in chloroform) were deposited 1 \( \mu \text{l} \) at a time by using a 25-\( \mu \text{l} \) Hamilton syringe (≈1.5 \( \mu \text{g/cm}^2 \)). Vehicle controls (chloroform) were performed simultaneously. Cell exposures were conducted for 1 h, unless otherwise stated. Immediately after exposure, the exposure media were aspirated, cells were washed once with PBS, the condi-
tioned medium was added back to each dish, and the cells were placed back in the CO$_2$ incubator for 1 h. This protocol was followed based on (1) time course studies that revealed greater injury at 2 h after exposure initiation, and 2) minimizing the period of serum absence while reducing cellular “shock” by the addition of fresh (unconditioned) growth media.

Acute Cell Injury Analysis

Analysis of total and injured cells utilized the differential permeability of the DNA fluorescent dyes EthD-1 (injured) and DAPI (total) (29). EthD-1, which intercalates and binds to DNA only if cell membranes are permeable, was applied first while DAPI was applied after cells were permeabilized by fixation. After the 1-h postexposure period, conditioned media were aspirated, and cells were treated with 4 μM EthD-1 (in PBS) for 20 min (in the dark, room temperature), fixed with 2% paraformaldehyde (pH 7.4 in PBS) for 20 min, and subsequently treated with 25 μM DAPI (in PBS containing 0.2% Triton X-100) for 20 min, and coverslips were mounted by using Vectashield (Vector Laboratories, Burlingame, CA).

Fluorescence analyses were conducted by using a Nikon Diaphot 300 fluorescence microscope equipped with a high-resolution digital camera (Princeton Instruments, Trenton, NJ). Fluorescent nuclei were counted by using MetaMorph image analysis software (version 4.01). Five random fields per dish (7) were counted based on studies indicating that similar results were obtained whether 5 or 10 fields per dish were counted. Areas near the dish walls and the dish midline sections were excluded to limit confounding from edge effects and potential differential media thicknesses (menisci) in dishes without reduced γ. During each exposure, dishes were run in duplicate for each experimental group and identified numerically to limit bias in evaluating cell injury. Experiments were repeated at least three times. For presentation, images were colored to their respective fluorescent hues by using Adobe Photoshop.

Data Analysis

All values are expressed as means ± 1 SD. Mean differences between groups were tested by ANOVA and Dunnett’s test post hoc (39). Significance was defined as $P < 0.05$.

RESULTS

O$_3$ Absorption Studies

Effect of DPPC monolayers on O$_3$ absorption. DPPC monolayers were deposited onto 250 μM AH$_2$ solutions (containing 50 μM DFX to limit autooxidation) and exposed to O$_3$ under quasi-steady-state conditions. Interfacial deposition of chloroform with or without DPPC (0.1 μg/cm$^2$) produced no alterations in O$_3$ absorption. Because uncompressed films did not affect O$_3$ absorption rates, all subsequent data were compared relative to control solutions containing no deposited lipid. As demonstrated in Fig. 2, when the DPPC monolayers were cyclically compressed and reexpanded, O$_3$ absorption rates were directly coupled to the extent of surface compression (γ). Following three compression-expansion cycles, monolayers were compressed from an initial area of 384.4 cm$^2$ (molecular area = 121 Å$^2$/molecule) to four stop points of 221, 200, 161, and 139 cm$^2$ (70, 62, 51, and 44 Å$^2$/molecule, respectively). Approximately 5 min were allowed at each compression point to permit determination of O$_3$ absorption as well as measurement of stable γ. O$_3$ uptake decreased linearly with decreasing γ (Fig. 3, $r^2 = 0.97$), attaining a maximal reduction of 62% ($P < 0.05$).

The γ dependence. Previous detergent studies (Tween 80) demonstrated that γ per se was not responsible for the observed monolayer-induced reductions in NO$_2$ uptake (10). Herein, SDS was utilized as even low concentrations of Tween 80 produced appreciable O$_3$ reactive absorption. A 0.01% SDS solution satisfied the criteria of being unreactive with O$_3$ and significantly reducing γ from 71.2 ± 2.6 to 50.8 ± 3.4 mN/m. Despite lowering γ, SDS failed to diminish the rate of AH$_2$-mediated O$_3$ absorption (250 μM AH$_2$: 5.11 ± 0.09 ng·min$^{-1}$·cm$^{-2}$; AH$_2$ + SDS, 5.06 ± 0.11 ng·min$^{-1}$·cm$^{-2}$). However, when uncompressed DPPC films were deposited within exposure flasks onto SDS solutions at sufficient concentration (0.5 μg DPPC/cm$^2$) to spontaneously reduce the γ (27.2 ± 0.2 mN/m) beyond that achieved by SDS alone, O$_3$ absorption was significantly decreased (47%) to 2.6 ± 0.06 ng·min$^{-1}$·cm$^{-2}$.

Gas-phase O$_3$ and aqueous substrate concentration dependence. AH$_2$ solutions with or without compressed DPPC monolayers were exposed to 0.3–1.1 ppm O$_3$ for 20 min. In the absence of a compressed DPPC film, total O$_3$ uptake rose sharply and was directly proportional to increasing initial O$_3$.
surface tension. At the end of each experiment, on surface reexpansion, \( [O_3] \) exit returned to baseline levels. Values are means ± SD for \( n \geq 5 \).

The effects of DPPC films on \( O_3 \) uptake across varying aqueous substrate concentrations and compositions (AH2, UA, or GSH) were evaluated. Similar to previous studies (4, 23, 28), \( O_3 \) uptake increased concomitant with aqueous AH2 concentrations in the absence of compressed films. Compression of the interfacial film significantly (\( P < 0.05 \)) constrained absorption rates to a relatively constant level (\( -0.10 \) ng·min\(^{-1}\)·cm\(^{-2}\)), despite substantial increases in the AH2 concentration (0.025–1 mM; Table 1). Based on studies demonstrating that AH2, UA, and GSH differentially drove \( O_3 \) reactive absorption (9, 10, 35), we also evaluated the relative influence of DPPC films across differing aqueous substrates. Despite differences in the baseline uptake, compressed films produced equivalent uptakes (average absorption rate of 0.98 ± 0.13 ng·min\(^{-1}\)·cm\(^{-2}\)) among the differing substrates (Table 1).

Effect of monolayer composition on \( O_3 \) absorption. Monolayers of varying phospholipid unsaturation and polar head group (POPC, POPG, and DLPC), in addition to BALF extracts, were examined to evaluate the effects of film composition on \( O_3 \) absorption (Table 2). Data are presented as \( O_3 \) fractional uptakes. The variation in the control fractional uptakes between POPC and POPG (1 mM GSH as the absorption substrate) was due to differences in the rate of \( O_3 \) delivery. Despite reducing \( \gamma \), maximally compressed POPC and POPG films (33.8 ± 1.4 and 34.6 ± 1.6 mM/m, respectively) did not alter \( O_3 \) absorption. Furthermore, \( \gamma \) increased to ~45 mM/m after 20 min of exposure. The more highly unsaturated phospholipid DLPC (full compression = \( \gamma \sim 43 \) mM/m) imparted no resistance to \( O_3 \) flux. Furthermore, \( \gamma \) increased more rapidly than with either POPC or POPG, in concert with the more facile \( O_3 \) reaction that occurs with polyunsaturated fatty acyl chains (28, 29, 31). Importantly, BALF extract films (\( \gamma \sim 54 \) mN/m) imparted a significant reduction in \( O_3 \) absorption, despite the relatively modest initial reductions in \( \gamma \).

Cell Culture Studies

Effects of aqueous phase conditions on acute cell injury. Human embryonic lung fibroblast cells (LU cells) (1, 6) were used expressly as targets to assess acute toxicity. These cells were chosen specifically because of their minimal secretory activity and relatively minor effects on media AH2 concentrations during 1-h air exposures (see discussion). Initial studies determined oxidant sensitivity under the employed culture conditions and the aqueous phase conditions necessary to generate acute cellular injury during \( O_3 \) exposure. We limited the exposure period to help maintain the antioxidant concentrations in the overlying media and to maintain stability of the phospholipid films in the subsequent DPPC studies. Log growth cells were overlain with MEM or MEM + H\(_2\)O\(_2\) (500 \( \mu \)M initial concentration), and, after 60-min incubation, the exposure media were replaced with conditioned media, and the cells were treated with fluorochromes 1 h later. Under these conditions, we observed H\(_2\)O\(_2\)-mediated EthD-1 staining and a concomitant loss of cells from the dishes (Table 3), demonstrating oxidant sensitivity comparable to other cell types (2, 27, 37) and suggesting that oxidant exposure induced both increased membrane permeability (cell injury) and cellular detachment.

Subsequently, log growth LU cells were exposed to \( O_3 \) by using a tilting exposure regimen. By tilting the culture dishes, the diffusional transport of solute \( O_3 \) and/or its reaction products to the cells was facilitated by introducing an intermittent thin film covering the upper population of cells. To induce demonstrable cell injury within a 1-h exposure period, it was necessary to employ very high \( O_3 \) concentrations, despite the
tilting regimen. When LU cells were exposed to ≤2.5 ppm O₃ in the absence of a reactive substrate, acute cellular injury was not detected by the employed methods (Table 3; Fig. 5, A1 and A3). However, exposure to 2.5 ppm O₃ in the presence of 500 μM AH₂ (60 min, 2 min/side tilting; 60-min postexposure time) produced significant cell injury (Table 3; Fig. 5, A2 and A4). Similar to the H₂O₂ studies, the exposure also led to cellular detachment from the culture dish, resulting in reduced cell densities at the time of fluorescent probe application. Analysis of the exposure MEM and postexposure conditioned media revealed the presence of intact cells, EthD-1-permeable cells, and cellular debris (data not shown), not dissimilar to what is observed in BALF recovered from acutely exposed animals. If one considers that cells lost from the dish (detached) also represent injury, then the total extent of cell injury can be computed as ~80% rather than the more conservative estimate shown in Table 3. These results suggest limitations in O₃ penetration to the cells and that reaction with AH₂ generated diffusible products capable of inducing acute injury.

**Effect of DPPC films on acute cell injury.** To perform acute cell injury studies with DPPC films, we employed an untilted regimen, which maintained an unstirred media interface for film stability. We also employed higher O₃ concentrations to overcome the diffusion and reaction limitations imposed by the greater volume of media required to provide a sufficient depth for phosphatidylcholine film deposition without perturbing the underlying cells. Log growth cells were overlain with 0.048 ml/cm² MEM-50 μM DFX ± 500 μM AH₂, DPPC (or chloroform alone) was deposited onto the aqueous phases after dishes were placed in the exposure chambers, and then cells were exposed to 4.4 ppm O₃ or 95% air-5% CO₂ for 1 h. Particular care was needed during film deposition to prevent the chloroform vehicle from contacting the cells, which alone (vehicle) showed no apparent cellular toxicity different from air-MEM or O₃-MEM. As presented in Table 4, similar to the tilted studies, O₃ exposure at even these very high concentrations produced no detectable injury in the absence of an aqueous reactive substrate. However, addition of AH₂ during exposure led to appreciable cell injury and cellular detachment, resulting in a diminution of the overall cell numbers (Fig. 5, B1 and B3). Addition of DPPC films alone did not influence exposure-induced cell injury with MEM-DFX alone. Notably, with the addition of AH₂, DPPC significantly reduced the extent of cell injury relative to the AH₂ systems lacking interfacial DPPC films (Table 4; Fig. 5, B2 and B4). In a separate series of experiments for γ measurements, cell cultures were exposed at 25°C outside of the incubators. Although γ were initially reduced to 25.0 ± 1.7 mN/m, they increased steadily over the exposure period to 44 ± 2.1 mN/m. Contamination of the interface by cellular debris likely contributed to the observed rise in γ. It is reasonable to speculate that the γ change was similarly affected during exposures conducted in the 37°C incubator. Despite the modest and nonstable reductions in γ, interfacial DPPC films limited O₃-induced cytotoxicity in the presence of aqueous reactive substrates in vitro.

**DISCUSSION**

Our previous studies (10), which documented that compressed lipid films introduced a resistive barrier to NO₂ reactive absorption, suggested that interfacial physicochemical conditions could affect delivery of exposure-related toxics to the underlying epithelium. Additional studies have shown that a remarkably heterogeneous pattern of pulmonary epithelial injury occurs within a short time after initiating O₃ exposure (21, 29, 37), further suggesting that surface conditions

---

**Table 1. Effect of substrate concentration, composition, and DPPC film compression on O₃ fractional uptake**

<table>
<thead>
<tr>
<th>AH₂ (±50 μM DFX)</th>
<th>25 μM</th>
<th>250 μM</th>
<th>1 mM</th>
<th>1 mM GSH</th>
<th>250 μM Uric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.45±0.05</td>
<td>0.72±0.03</td>
<td>0.84±0.02</td>
<td>0.42±0.02</td>
<td>0.73±0.02</td>
</tr>
<tr>
<td>Compressed DPPC</td>
<td>0.16±0.08*</td>
<td>0.18±0.04*</td>
<td>0.20±0.02*</td>
<td>0.13±0.04*</td>
<td>0.14±0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SD. AH₂, ascorbic acid; DFX, desferrioxamine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. Ozone (O₃) fractional uptakes were computed from the mass balance across the exposure probe (see MATERIALS AND METHODS) during exposures to 0.8 parts/million (ppm) O₃ for 20 min (25°C). Control refers to buffer + solute-only aqueous systems with or without chloroform overlay. DPPC refers to systems wherein 0.1 μg/cm² DPPC was initially deposited and the interface compressed by ~62% to yield surface tension of ~25 mN/m. *Statistically significant (P<0.05) decline in O₃ absorption relative to substrate concentration-matched controls (n=5).

**Table 2. Effect of interfacial film lipid composition on O₃ fractional uptake**

<table>
<thead>
<tr>
<th>1 mM GSH</th>
<th>250 μM AH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>0.57±0.05</td>
</tr>
<tr>
<td>POPG</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>DPPC</td>
<td>0.59±0.03</td>
</tr>
<tr>
<td>BALF Extract</td>
<td>0.86±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; BALF, bronchoalveolar lavage. O₃ fractional uptakes were computed from the mass balance across the exposure probe (see MATERIALS AND METHODS) during exposures to 0.8 ppm O₃ for 20 min (25°C). Approximately 0.1 μg/cm² POPC or POPG was spread on solutions containing 250 μM AH₂ or 50 μM DFX. *Statistically significant (P<0.05) decline in O₃ absorption relative to substrate-matched, nonlipid controls (n=5).

**Table 3. Exposure-induced cytotoxicity in cultured LU cells: cell injury induced during H₂O₂ or O₃ + tilting regimens**

<table>
<thead>
<tr>
<th>Cells/field</th>
<th>H₂O₂*</th>
<th>Air-MEM†</th>
<th>Air-AH₂†</th>
<th>O₃-MEM†</th>
<th>O₃-AH₂†</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Injured</td>
<td>&lt;1</td>
<td>34‡</td>
<td>68±15</td>
<td>64±16</td>
<td>61±19</td>
</tr>
</tbody>
</table>

Values for cells/field are means ± SD. *LU cells were grown to ~30% confluency, covered with 1 ml MEM, and incubated in the presence or absence of 500 μM H₂O₂ (initial concentration). †Approximately 40–50% confluent cells were covered with 1 ml MEM-50 μM DFX with or without 500 μM AH₂ and exposed to 95% air-5% CO₂ or 2.5 ppm O₃ for 60 min with cyclic tilting (37°C). ‡Statistically significant (P<0.05) change from either the matched H₂O₂ controls or from the air-MEM controls.
The inherent inability to reproducibly control and modulate pulmonary interfacial and ELF conditions dictated the use of in vitro models and their recognized limitations. These included our lack of ability to fully compress the interfacial films, the oversimplification of the physicochemical conditions within the ELF and at the gas-liquid interface, the need to conduct studies within relatively brief time spans, the use of heavily deposited rather than compressed films for the cell studies, and the use of nonepithelial cells. However, our approaches facilitated measures of O$_3$ flux through defined interfacial films (Fig. 1), film influences across gas- and aqueous-phase conditions, and determination of the impact of surface fluid thickness, solute chemistry, and altered O$_3$ flux on exposure-mediated acute cell injury.

As demonstrated in Fig. 2, we observed a very strong association between surface compression and O$_3$ uptake, suggesting that the resistive properties may vary as $\gamma$ changes among anatomic sites and with lung inflation. Although O$_3$ uptake reductions were coupled to surface compression (Fig. 3), the proportional decline in O$_3$ flux clearly could not continue down to the $\gamma$ occurring within the alveoli ($\approx$10 mN/m). Furthermore, compressed DPPC films almost completely abrogated the relationships among absorption rates and gas-phase O$_3$ concentrations (Fig. 4), aqueous substrate concentrations, or substrate composition (Table 1). Because proximal alveolar damage unequivocally occurs and is sensitive to the inspired concentration, some O$_3$ penetration through the interfacial film must occur, even with very low $\gamma$. This may be attributable to variations in interfacial phospholipid composition, ELF substrate composition, and/or topography of the lung surface.

Both the osmiophilic layers and reduced $\gamma$ ($\approx$25 mN/m) support the fact that conducting airways contain interfacial surface-active lipids. Although DPPC is the principal surface-active component of alveolar surfactant (9, 40), the precise composition of the airway interfacial lipids has not been completely resolved. Airway $\gamma$ may be elevated, relative to the alveoli, due to the presence of unsaturated phospholipids and/or other biochemical moieties. Compression of pure unsaturated phospholipid films did not inhibit O$_3$ flux, despite their initial lowering of the $\gamma$ (Table 2). Ozonation of unsaturated fatty acids leads to alterations in the packing order, fluidity, and polarity of unilamellar vesicle membranes (36). Such chemical and biophysical alterations could have accounted for the $\gamma$ increases observed during exposure. Furthermore, rapid film disruption may have accounted for unsaturated phospholipid films not inhibiting O$_3$. Detecting film reac-

Table 4. Exposure-induced cytotoxicity in cultured LU cells: cell injury induced during untilted regimens

<table>
<thead>
<tr>
<th>Cells/field</th>
<th>Air-MEM ± DPPC</th>
<th>Air-AH$_2$ ± DPPC</th>
<th>O$_3$-MEM ± DPPC</th>
<th>O$_3$-AH$_2$ ± DPPC</th>
<th>O$_3$-AH$_2$ + DPPC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Injured</td>
<td>54±9</td>
<td>49±4</td>
<td>44±8</td>
<td>37±4†</td>
<td>35±11†</td>
</tr>
</tbody>
</table>

Values for cells/field are means ± SD. Approximately 35% confluent cells were covered with 0.46 ml MEM-50 μM DFX with or without 500 μM AH$_2$ and exposed to 95% air-5% CO$_2$ or 4.4 ppm O$_3$ for 60 min without tilting. DPPC was deposited at $-1.5$ μg/cm$^2$, which reduced surface tensions to $-25$ mN/m. *No differences were observed due to the presence (+) or absence (−) of the DPPC films. †Statistically significant (P<0.05) change from the air-MEM controls.
tion with O₃ could not be accomplished because the absolute number of unsaturated bonds reacting with O₃ would have been extremely small relative to the total flow of O₃ through the exposure apparatus.

Importantly, even a relatively crude ELF extract obtained from whole lung lavage, which represented an admixture of airway and alveolar materials, produced significant resistance to O₃ flux. Surface interactions of inhaled O₃ are complex, whereas the gas-phase concentration likely does not appreciably vary within any one airway generation, very focal lesions occur. Furthermore, lesion foci appear within progressively distal daughter branches, even though there is a longitudinal decline in the air space O₃ concentration. The heterogeneous pattern of O₃-induced acute airway epithelial injury (21, 29, 37) likely results from combinations of airflow patterns, interfacial conditions, O₃ reaction with ELF interfacial and fluid-phase substrates, ELF composition, and ELF regulation within microenvironmental regions of the conducting airway tree. Although it is difficult to directly extrapolate the observations obtained herein, it may be reasonable to speculate that the presence of interfacial lipid films modulates the intrapulmonary dispersion of inhaled O₃ and may contribute to the focal pattern of airway injury.

Use of the LU cells was based on the fact that their relative metabolic and secretory activities facilitated control of extracellular conditions and on our ability to quantify acute cell injury under the employed exposure conditions. Other cell types may display greater sensitivity, but, for these initial studies, we felt it critical to maintain maximal control of the extracellular conditions. We investigated the time dependence of 500 µM AH2 media disappearance with or without 50 µM DFX and/or O₃ exposure. Under control conditions (95% air-5% CO₂, MEM), after 60 min, we observed ~50% decline in AH2 concentration without DFX but only ~25% loss with DFX. O₃ exposure increased AH2 loss to ~44% with DFX but >70% when DFX was absent. Furthermore, we also observed a modest degree of acute cell injury in control cells incubated for 60 min with 500 µM AH2 in the absence of DFX, suggesting adventitious Fe initiated redox production of cytotoxic reactive species, thus further dictating the use of DFX. We employed log-growth cultures, as, under these conditions, the LU cells displayed enhanced oxidant sensitivity but somewhat large cell number variances during random field analysis (Table 3). Although the LU cells displayed both O₃ and H₂O₂ susceptibility, and despite the use of log-growth cells, it still required very high oxidant concentrations within the 1-h time span to induce appreciable cytotoxicity.

Because we detected no differences among air-MEM, air-MEM-AH₂, and O₃-MEM exposures (all containing DFX), cell injury was predominantly related to O₃ reaction with aqueous reactive substrates rather than O₂ diffusion through the overlying media. To our knowledge, this is the first demonstration that O₃ reaction with antioxidants leads to the apparent production of acutely cytotoxic product(s) (Table 3, Fig. 5A). Whereas the precise chemistry of the O₃ + AH₂ reaction has not been completely characterized, production of the O₂ radical (O₂•) or other secondary oxidant(s) is possible (33). Thus, as has been demonstrated for lipid ozonation products (11, 13, 28), constituent concentrations may appreciably influence the production of secondary toxicants within the ELF milieu and, therefore, may, in part, mediate the extent of cell injury. Consequently, there is a clear need to explore the complexities among O₃ exposure, airway anatomy, constituents of the ELF, and the induction of acute cell injury.

Pryor (32), Hu et al. (19), and Postlethwait and Ultman (31), using simplified reaction and diffusion analyses, have estimated that O₃ may only penetrate ~0.1 µm into the ELF. However, these analyses have used initial conditions wherein reactive substrate concentrations implicitly remain high so that reaction predominates over diffusion throughout exposure. Recent observations suggest that local ELF concentrations may fall during the early phases of exposure (E. M. Postlethwait, personal communication; Ref. 26). Furthermore, we have noted in NO₂ reaction and diffusion studies that, if substrate concentrations fall below critical thresholds, NO₂ diffusion through an aqueous layer is sufficient to induce membrane protein nitration (43). Consequently, direct contact between solute O₃ and the apical cell surfaces cannot be completely ruled out. Nonetheless, if this occurred, it was not sufficient to induce detectable cell injury in these studies.

To facilitate spreading and maintenance of DPPC films, cell cultures required a thicker overlying media layer and an untiiled exposure regimen. Because we were unable to observe changes in O₃ flux due to unsaturated phosphatidylcholine films, we did not explore their effects on exposure-mediated acute toxicity. Under the employed conditions, we were able to induce acute cell injury only via the use of extreme gas-phase concentrations and AH₂. As during the tilting regimens, we detected no differences among air-MEM, air-MEM + chloroform, air-MEM + AH₂, O₃-MEM + chloroform, and O₃-MEM (all with DFX; Table 4). DPPC films also produced no discernable influence on exposure-related injury in the absence of an absorption substrate. However, the addition of AH₂ led to a significant increase in the percentage of injured cells, although the magnitude of cellular detachment and remaining injured cells was somewhat less than during the tilting regimens. The media layer thickness likely further restricted O₃ diffusion and facilitated quenching of the O₃ + AH₂ cytotoxic product(s). Media depth also influenced the extent of NO₂-induced perturbations in primary cultures of rat alveolar type II cells (8).

Application of DPPC films significantly reduced the percentage of injured cells resulting from O₃ + AH₂ exposures (Table 4). However, despite the presence of the DPPC films, cellular detachment occurred, suggesting that exposure-related perturbations had not been abrogated but only reduced in magnitude. If one considers the detached cells as injured, the O₃-AH₂ without DPPC cultures experienced ~42% injury, whereas addition of the DPPC film (O₃-AH₂ + DPPC) significantly reduced injury to ~30%. Due to the inability to perform surface compressions on the cell cultures during O₃ exposure, the DPPC films were deposited at high density, which also imparts a resistive barrier (10). Whether the barrier properties were maintained throughout the cell exposures is difficult to ascertain, because γ measurements could not be performed within the incubator apparatus. However, cell injury at a comparatively lesser but still appreciable rate could have occurred if the films were disrupted due to cellular detachment and/or release of cel-
PHOSPHOLIPID INHIBITION OF O₃ CELL INJURY

L1177

ular constituents. This would have led to a coupled increase in O₃ flux and a temporal shift in cytotoxicity. Such scenarios are likely because we observed a rise in γ during exposures conducted at room temperature.

In conclusion, we regard these as initial observations regarding the potential for in situ interfacial films to modulate dose-effect relationships within the lung. Due to the strict in vitro nature of these current studies, it is premature to formulate dogmatic conclusions regarding the absolute extent that surface-active films and the underlying fluid may affect the relationships between intrapulmonary gas-phase O₃ concentrations and cellular responses. Nonetheless, the data presented herein suggest that the potential resistive properties introduced by surface films may appreciably alter the rate of toxic product formation relative to a given gas-phase concentration. These parameters must be carefully controlled for during cell culture exposures to mimic the conditions that occur in vivo, and biological responses observed in vitro should be extrapolated with caution.

ACKNOWLEDGMENTS

The authors thank Robert Rahimi for valuable assistance with the cell culture exposures.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-54696 (to E. M. Postlethwait).

REFERENCES