Formation of Biologically Active Oxysterols During
Ozonolysis of Cholesterol Present in Lung Surfactant

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ABSTRACT

Exposure of the lung to concentrations of ozone found in ambient air is known to cause toxicity to the epithelial cells of the lung. Because of the chemical reactivity of ozone, it likely reacts with target molecules in pulmonary surfactant, a lipid rich material which lines the epithelial cells in the airways. Phospholipids containing unsaturated fatty acyl groups and cholesterol would be susceptible to attack by ozone, which may lead to the formation of cytotoxic products. While free radical derived oxidized cholesterol products have been frequently studied for their cytotoxic effects, ozonized cholesterol products have not been studied, though they could reasonably play a role in the toxicity of ozone. The reaction of ozone with cholesterol yielded a complex series of products including 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, 5-hydroperoxy-B-homo-6-oxa-cholestan-3β,7a-diol and 5β,6β-epoxycholesterol. Mass spectrometry and radioactive monitoring were used to identify the major cholesterol derived product during the reaction of 2 ppm ozone in surfactant as 5β,6β-epoxycholesterol, which is only a minor product during ozonolysis of cholesterol in solution. A dose dependent formation of 5β,6β-epoxycholesterol was also seen during direct exposure of intact cultured human bronchial epithelial cells (16-HBE) to ozone. Studies of the metabolism of this epoxide in lung epithelial cells yielded small amounts of the expected metabolite, cholestan-3β,5α,6β-triol, and more abundant levels of an unexpected metabolite, cholestan-6-oxo-3β,5α-diol. Both 5β,6β-epoxycholesterol and cholestan-6-oxo-3β,5α-diol were shown to be cytotoxic to cultured 16-HBE cells. A possible mechanism for cytotoxicity is the ability of these oxysterols to inhibit isoprenoid based cholesterol biosynthesis in these cells.
Abbreviations:

α-epoxide, 5α,6α-epoxycholesterol; β-epoxide, 5β,6β-epoxycholesterol; 3,5,6-triol, cholestan-3β,5α,6β-triol; secosterol, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; 6-oxo-3,5-diol, cholestan-6-oxo-3,5-diol; BSTFA, Bis(trimethylsilyl)fluoroacetamide; 1-palmitoyl-2-oleoyl-glycerophosphocholine, 16:0a/18:1-GPCho; 1-palmitoyl-2-(9′-oxo-nonanoyl)-glycerophosphocholine, 16:0a/9al-GPCho; d₃-platelet activating factor, d₃-PAF; d₆-dipalmitoyl-glycerophosphocholine, d₆-16:0a/16:0-GPCho
INTRODUCTION

Human exposure to 0.2 ppm levels of ozone in ambient air has been shown to cause numerous pulmonary effects such as increased airway inflammation and decreased pulmonary function (1,2). Studies of ozone in animals using up to 3 ppm ozone have been shown to cause increased airway hyperresponsiveness and epithelial cell death. It has been hypothesized that the very high chemical reactivity of ozone limits the distribution of this gas in the pulmonary system, preventing direct exposure to the cellular components of the lung. In part ozone may react with the various components of the epithelial cell lining fluid in the lung, also known as pulmonary surfactant, which includes proteins, lipids and single electron antioxidant agents such as ascorbic acid (3-5). Due to the very high reactivity of ozone with lipids containing double bonds, considerable emphasis has been placed on the reaction of ozone with lipid compounds in the lungs and the possibility that the adverse effects of ozone are mediated by lipid ozonized products (6). Evidence in support of this theory has been accumulating with the identification of biologically active phospholipids (7) such as 1-hexadecanoyl-2-(9-oxo-nonanoyl)-glycerophosphocholine, found following ozone exposure to lung surfactant (8). This oxidized phospholipid which eluted as a somewhat polar product on normal phase HPLC was found to initiate apoptotic death in monocytes and macrophages. However, a relatively non-polar component was also found to elute from this normal phase HPLC separation that was also cytotoxic, and preliminary data suggested that several oxidized neutral lipid products were present in this fraction.

Cholesterol is the most abundant neutral lipid present in pulmonary surfactant and this molecule has a double bond that would be susceptible to attack by ozone (9,10). While there has been some controversy about the exact chemical structure of the major ozonolysis product when cholesterol is ozonized in solution at high ozone concentrations (>0.1%) (11-14), electrospray
tandem mass spectrometry was recently used to characterize this chemically reactive cholesterol ozonolysis product as 5-hydroperoxy-B-homo-6-oxa-cholestane-3β,7a-diol (Scheme 1) (15). Reduction of 5-hydroperoxy-B-homo-6-oxa-cholestan-3β,7a-diol has been shown to primarily yield 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (5,6-secosterol) (11-13). In fact, this reduced product has been detected in lung homogenate and bronchoalveolar lavage fluid of rats exposed to ozone, and it has thus been suggested as a biomarker for ozone exposure (16). The recent observation of 5,6-secosterol in arterial plaques has provided evidence that the formation of ozone may also occur as the result of normal biochemical events taking place in vivo during an inflammatory response (17).

It is important to consider that the interaction between ozone and cholesterol has primarily been studied in organic solvents with high levels of ozone, where 5-hydroperoxy-B-homo-6-oxa-cholestan-3β,7a-diol is the major product (11,14,15). However, environmentally relevant concentrations of ozone acting on lipid cellular membranes or in lipid rich pulmonary surfactant could involve different chemistry, because of the ordered nature of the lipid bilayer, yielding alternate products. Isolated bronchoalveolar lavage fluid was exposed in vitro to precise levels of ozone in a carefully controlled ozone chamber to study the formation of cholesterol derived ozonolysis products. This revealed the formation of 5β,6β-epoxycholesterol (β-epoxide) (Scheme 1) as a more abundant product than 5-hydroperoxy-B-homo-6-oxa-cholestane-3β,7a-diol in this system. The ability of this compound and its cellular metabolites to cause cytotoxicity and to inhibit cholesterol synthesis in cultured human bronchial epithelial cells was subsequently studied.
METHODS

Materials. Cholesterol (>99%) was purchased from Sigma (St. Louis, MO). Radioactive [4-\textsuperscript{14}C] cholesterol (45-60 mCi/mmol dissolved in ethanol) was purchased from NEN (Boston, MA). Radioactive [1-\textsuperscript{14}C] acetate (56.6 mCi/mmol dissolved in ethanol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Stable isotope labeled 2,2,3,4,4,6-d\textsubscript{6}-cholesterol (98% atom percent, excess d\textsubscript{6}) was purchased from Cambridge Isotope Laboratories (Andover, MA). Solvents, cell culture media, and culture plates were purchased from Fisher Chemical (Pittsburgh, PA). Bis(trimethylsilyl)fluoroacetamide (BSTFA) and trypan blue dye (0.4%) were purchase from Sigma. Bis(trimethyl-d\textsubscript{5}-silyl)acetamide (99%) was purchased from Isotech (Miamisburg, OH). Rat lung lavage fluid was provided by Dennis Voelker (National Jewish Medical and Research Center, Denver, CO).

Identification of Cholesterol Ozonolysis Products in Lung Surfactant. Bronchoalveolar lavage fluid from rats was concentrated by centrifugation at 20,000 x g for 1 hr in 5 mM CaCl\textsubscript{2} (18). The pellet was resuspended in phosphate buffered saline to a final cholesterol concentration of 30 \( \mu \text{g/ml} \) cholesterol. For identification of cholesterol metabolites, 2.5 \( \mu \text{l} \) of tracer [\textsuperscript{14}C] cholesterol was added to 1 ml of the lavage fluid for a final concentration of 200 nCi/ml (0.2% ethanol). Experiments were carried out in triplicate by ozonolysis of 100 \( \mu \text{l} \) of this labeled lavage fluid in 35 mm diameter tissue culture multiwell plates. The plates were exposed to various concentrations of ozone using a computer-controlled in vitro ozone exposure chamber. This system was capable of delivering precise concentrations of ozone from 0.1 ppm to 10 ppm in humidified air, as previously described (8).
After ozonolysis, the samples in each well were diluted with 2 ml of water, transferred to glass tubes, and wells were washed with 2 ml of methanol. To the transferred samples, 3 ml methylene chloride was added and lipids extracted essentially as described by Bligh and Dyer (19). After drying the extract with a stream of dry nitrogen, the products were dissolved in 100 µl ethanol, then injected onto a C18 (250 x 4.6 mm) reversed phase column (Phenomenex, Torrance, CA) at a flow rate of 1 mL/min. Solvent A was methanol:water:acetonitrile (v:v:v; 60:20:20) with 1 mM ammonium acetate; solvent B was methanol with 1 mM ammonium acetate. The gradient ran from 50% to 100% B in 20 min and stayed at 100% B for 20 min. Radioactive monitoring coupled with mass spectrometry was used to detect the products of cholesterol ozonolysis as previously described (15).

Quantitation of Epoxycholesterol, Cholesterol and Phospholipids. Both the alpha and beta isomers of 5,6-epoxycholesterol were synthesized based on the method of Sevanian et al. (20) with slight modifications. Briefly, 19.1 mg chloroperoxybenzoic acid was added to 38.6 mg cholesterol in 12 mL methylene chloride and stirred overnight at 4°C. Deuterated (2,2,3,4,4,6-H6) cholesterol was used for synthesis of deuterated 5,6-epoxycholesterol. The solution was washed three times with water, and then with a saturated salt solution. Finally, the solvent was evaporated using a roto-evaporator and the product was resuspended in ethanol. Separation of the alpha and beta isomers was achieved using RP-HPLC on an Alltech column (250 x 10.0 mm, C18; Deerfield, IL) at a flow rate of 4 mL/min with the gradient described above. Fractions were collected (1 min) and dried under vacuum; after weighing, the fractions were derivatized with BSTFA and analyzed by GC/MS to determine purity of the reference standards (>95%).
Quantitation of both cholesterol and β-epoxide was carried out using stable isotope dilution mass spectrometry. Deuterated β-epoxide (40 ng in 25 μL) was added to each point of the standard curve, and to all samples before extraction with iso-octane. An identical protocol was used to determine total cholesterol. Samples (10% aliquot) were introduced onto the mass spectrometer using a 150 x 1.0 mm Columbus C18 column (Phenomenex; Rancho Palos Verdes, CA). The solvent system used was the same as for identification of cholesterol ozonolysis products, except a modified gradient was used, with 75% to 100%B for 10 min followed by 100%B for 20 min. Multiple reaction monitoring analysis was carried out on a Sciex API-2000 mass spectrometer (Perkin-Elmer Life Sciences, Thornhill, Ontario, Canada). Positive ion mode was used with an ionspray voltage of 4500 V, declustering potential of 40 V, focusing potential 350 V and collision energy of 12 V. Nitrogen was used in the collision cell with a collision gas thickness of $2.17 \times 10^{15}$ mol/cm$^2$. The transitions monitored were m/z 420 $\rightarrow$ 385 for α-epoxide and β-epoxide, m/z 426 $\rightarrow$ 391 for d$_{-}$β-epoxide, m/z 404 $\rightarrow$ 369 for cholesterol, m/z 436 $\rightarrow$ 383 for 5,6-secosterol and 6-oxo-3,5-diol, m/z 438 $\rightarrow$ 385 for 3,5,6-triol. The dwell time for each transition was 800ms. The standard curve was linear for the range tested, from 0.625 ng to 320 ng 5β,6β-epoxycholesterol and from 100 ng to 6.4 μg cholesterol.

Quantitation of 1-palmitoyl-2-oleoyl-glycerophosphocholine (16:0a/18:1-GPCho) and 1-palmitoyl-2-(9’-oxo-nonanoyl)-glycerophosphocholine (16:0a/9al-GPCho) in 75μl aliquots of rat BAL exposed to 0.2 ppm ozone was achieved by addition of 20 ng d$_{-}$-platelet activating factor (d$_{-}$-PAF) and 320 ng d$_{x}$-dipalmitoyl-glycerophosphocholine (d$_{x}$-16:0a/16:0-GPCho) at the same time as the cholesterol and β-epoxide internal standards. After neutral lipid extraction of the samples with iso-octane (supra vide), 2 ml methylene chloride was added to the remaining H$_2$O:MeOH layer to extract the phospholipids. The organic layer was dried under nitrogen and solvated in 500 μl
normal phase solvent A (Hexane:Isopropanol:H₂O; 30:40; v:v) for injection onto a 150 x 1.0 mm Prodigy silica column (Phenomenex; Rancho Palos Verdes, CA). Initial conditions were 75% solvent B (Hexane:Isopropanol:H₂O; 30:40:7; v:v:v; 1% ammonium acetate). The gradient increased to 100% B over 6 min, and stayed constant for another 30 min. The mass spectrometer was run in positive ion mode with a higher declustering potential (100 V) and a higher collision energy (40 V) than used when monitoring cholesterol derived products. The transitions monitored for the phospholipids were m/z 527 → 184 for d₃-PAF, m/z 650 → 184 for 16:0a/9αl-GPCho, m/z 740 → 184 for d₆-16:0a/16:0-GPCho, and m/z 760 → 184 for 16:0a/18:1-GPCho. The standard curve was linear for the range tested 20 ng to 640 ng 16:0a/9αl-GPCho and 80 ng to 2.56 μg 16:0a/18:1-GPCho.

**Cell Ozonolysis, Cytotoxicity and Inhibition of Cholesterol Synthesis.** The 16 HBE 14o⁻ (16-HBE) human bronchial epithelial cell line (21) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin on uncoated 6- or 24-well culture dishes. Cells were grown at 37°C in a 95% air, 5% CO₂ incubator with 100% humidity. For ozonolysis, cells were grown to approximately 50% confluency in 6-well plates. Before ozonolysis the media was removed and cells were washed with PBS. A small volume (300 μl) of HBSS was added to each well to keep the cells from drying out during ozone exposure. After exposure cells were trypsinized and the lipids were extracted as described above.

Trypan blue exclusion was used to measure cytotoxicity (22). 16-HBE cells were grown in 24 well plates to >50% confluency (2 x 10⁵ cells) and then washed and covered with 1ml serum containing media per well. Ethanolic solutions of oxysterol or corresponding vehicle were added to cell media (0.5% ethanol final concentration), and the cells were incubated at 37°C for the duration of the exposure. At the indicated time points, cells were trypsinized and a small aliquot
was diluted 1:1 with a 0.4% trypan blue solution (final concentration 0.2%). At least 100 cells were counted per sample using a hemocytometer, and the percentage that incorporated the dye was calculated. No effect of vehicle was observed. For studies of protection by exogenous cholesterol, 1, 3, 10, or 30 μM cholesterol in ethanol was added immediately prior to treatment with oxysterol.

For studies of inhibition of cholesterol synthesis, the incorporation of radiolabeled acetate into cholesterol and neutral lipids was determined by modification of the method of Kandutsch et al. (23). Briefly, cells at 50% confluency in 6 well dishes were washed and fresh media (3 mL) was added. Cells were treated with indicated concentrations of oxysterols in ethanol for 24 hr, then 7 μCi [14C] acetate was added for 3 hr (final ethanol concentration 0.3%). Cells were trypsinized and lipids were extracted from the media and cells with 3 volumes iso-octane. The extract was dried with anhydrous magnesium sulfate to remove residual water soluble radioactive components. The iso-octane was then dried under N₂ and resuspended in 100 μl methylene chloride for TLC chromatography using silica gel G (Analtech; Newark, DE) activated for 2 hr at 110°C. The TLC solvent system employed was 99.5% ether with 0.5% ammonium acetate in which cholesterol and neutral lipids had a Rₜ of 0.61 and 0.83, respectively. The radioactive signal was quantitated by integration of the radioactive signal for the peak at each Rₜ using a Bioscan system 200 imaging scanner with Win-scan software (Bioscan, Washington, D.C.).

**Structural Characterization of the Metabolites.** Three 10 cm diameter plates of 16-HBE cells (approximately 30 million cells) were treated with 3 μM β-epoxide overnight (final ethanol concentration 0.03%). Samples were pooled and the lipids were extracted as described above prior to injection on a RP-HPLC column flowing at 1 ml/min with 50 μl eluate split to the mass spectrometer to monitor for the metabolite transition (m/z 436 → 383), and the remaining effluent
directed to a fraction collector (one minute fractions). A portion of the HPLC fraction containing the metabolite was infused into the API III instrument (Applied Biosystems, Thornhill, Ontario, Canada) for tandem mass spectrometric analysis, and the rest was dried under vacuum. The same method was used to acquire deuterated metabolites (cells were treated with deuterated \( \beta \)-epoxide under identical conditions).

In order to characterize the metabolites by electron ionization (70 eV) mass spectrometry, an aliquot of the metabolite or its deuterated analog was derivatized by addition of 50 \( \mu l \) acetonitrile and 50 \( \mu l \) BSTFA followed by a 15 min heating to 65°C for 15 min. An aliquot (2 \( \mu L \)) of each derivatization solution was analyzed by a gas chromatograph mass spectrometer using electron ionization at +70 eV (Trace 2000, Thermo-Finnigan, San Jose, CA). The temperature gradient ran from 150°C to 260°C at 20°C/min and 260°C to 310°C at 4°C/min on a 30 meter DB-1 column (Phenomenex, Torrance, CA) with a 0.25 mM ID and a 0.25 \( \mu \) stationary film thickness.

Sodium borohydride reduction of \( \alpha \)-epoxide, \( \beta \)-epoxide, and the unknown metabolite was carried out by adding an excess of \( \text{NaBH}_4 \) to the oxysterol dissolved in ethanol for 2 hr followed by iso-octane extraction. Epoxide hydrolysis to vicinal diols of \( \alpha \)-epoxide, \( \beta \)-epoxide, and the unknown metabolite was carried out by treating 5 \( \mu g \) of the oxysterol with 30 \( \mu l \) perchloric acid in 0.5 ml of THF: \( \text{H}_2\text{O} \):acetone (v:v:v; 4:1:0.5) and stirring for 4 hr at room temperature (24).

For large scale synthesis of cholestane-3,5,6-triol (3,5,6-triol), 10 mg \( \beta \)-epoxide was treated with 0.5 ml perchloric acid in 4 ml of THF: \( \text{H}_2\text{O} \):acetone (v:v:v; 4:1:0.5). The lipids were extracted with methylene chloride and the organic layer washed 3x with water. The 3,5,6-triol was purified by RP-HPLC as described above for \( \beta \)-epoxide. Cholestan-6-oxo-3,5-diol (6-oxo-3,5-diol) was synthesized by the method of Fieser and Rajagopalan (25). Briefly, 10 mg cholestanetriol was
dissolved in 4.5 ml ether, 750 μl methanol, and 750 μl water. N-bromosuccinimide (108 mg) was added and the reaction was stirred for 3 hr at room temperature. The solution was diluted with water and extracted with methylene chloride. The organic fraction was dried under vacuum and purified by RP-HPLC. The resulting product was derivatized with BSTFA and characterized by GC/MS.
RESULTS

Treatment of diluted calf lung surfactant extract with high concentrations of ozone was previously reported to generate several classes of biologically active products, two of which had very different polarity when separated by normal phase chromatography (8). When analyzed by electrospray mass spectrometry (LC/MS), the early eluting normal phase fraction that reduced monocyte viability was found to generate abundant positive ions at m/z 383, 401, 419, 435, and 452 (data not shown). These ions were consistent with various neutral lipids, including oxidized cholesterol, suggesting that cholesterol within pulmonary surfactant could be transformed into biologically active metabolites that mediated the toxicity of ozone. In order to unambiguously determine if components in lung surfactant could in fact be derived from cholesterol reacting with relevant concentrations of ozone, a trace amount of [14C]cholesterol was added to isolated rat BAL fluid and the solution was exposed to 2.0 ppm ozone in a controlled chamber for 4 hr. Lipids were then extracted, chromatographed by reversed phase HPLC, and the effluent analyzed by on-line mass spectrometry and radioactive scintillation detection. Two major radioactive products were observed along with unreacted cholesterol (Figure 1). The least lipophilic component (peak A) eluted at 16 min and generated an abundant ion at m/z 511 (negative ion mode) and at m/z 452 (positive ion mode). This component was identified as 5-hydroperoxy-B-homo-6-oxa-cholestan-3,7a-diol based on collision induced dissociation properties and RP-HPLC retention time in comparison with a previously identified product following the exposure of cholesterol to ozone in THF and water (15). The compound that eluted at 21 min (peak B) generated an abundant positive ion at m/z 420 by electrospray ionization that corresponded to an ammonium ion adduct of cholesterol with the addition of one oxygen atom, which agreed with a previously identified product of cholesterol ozonolysis, namely 5,6-epoxycholesterol (11). This oxidized cholesterol product had
been previously studied since it was observed to form during a very different type of oxidative stress, namely free radical mediated cholesterol peroxidation (26). Synthesis of both epoxide steroisomers had been previously described (20), which provided a facile means to synthesize deuterated 5α,6α-epoxycholesterol (α-epoxide) as well as deuterated β-epoxide. Each isomer was added in separate experiments to pulmonary surfactant after exposure to relatively low concentrations of ozone and it was found that the radioactive peak eluting at 21 min was in fact only one of the epimers, namely 5β,6β-epoxycholesterol based on co-elution of the deuterated β-epoxide with peak B (data not shown).

Deuterated β-epoxide was subsequently used as a mass spectrometry internal standard to facilitate quantitation of both isomers of epoxycholesterol in rat BAL treated with ozone. When BAL was exposed for 4 hr to increasing levels of 0.2, 0.5, and 1.0 ppm ozone, a dose dependent formation of β-epoxide was observed (Figure 2). Up to 200 ng of β-epoxide was observed to form under these conditions, while approximately 3 μg of unreacted cholesterol remained in the surfactant. The beta isomer was formed in preference to the alpha isomer at all ozone concentrations studied with a ratio of approximately 5:1. Samples of rat BAL exposed to filtered air had low but detectable levels of β-epoxide, however, α-epoxide was not detected.

Phospholipids that contain a double bond in a fatty acyl chain are also an abundant component of pulmonary surfactant, and therefore it was of interest to determine the abundance of cholesterol derived ozonolysis products relative to phospholipid derived ozonolysis products. Aliquots of surfactant containing 3.3 nmol of 16:0a/18:1-GPCho and 5.6 nmol of cholesterol contained 66 pmol of β-epoxide and 10 pmol of the phospholipid ozonolysis product, 16:0a/9al-GPCho previously studied (8). After exposure to 0.2 ppm ozone for 4 hr, the level of 16:0a/9al-GPCho increased to 42 pmol and β-epoxide increased to 184 pmol. Therefore there was a 3-4 fold
increase in the levels of both products following ozonolysis, suggesting that cholesterol is a relevant target for oxidation by ozone, and that cholesterol ozonolysis products are formed in similar abundance to phospholipid derived ozonolysis products.

The pulmonary surfactant layer in some areas of the lung may be sufficiently thin to permit direct exposure of underlying epithelial cells to ozone present in inspired air. This would be especially true for cells in the alveoli where surfactant sits mainly in the junctions between cells (27). Also, inflammatory cells such as macrophages could protrude out of the surfactant layer and thus have direct contact with ozone (6). Therefore, the presentation of cholesterol in the lipid environment of a cellular membrane was examined for the production of epoxycholesterol. Cultured human bronchial epithelial cells (16-HBE) were exposed directly to environmentally relevant concentrations of ozone (0.2, 0.5, 1.0 ppm) for 1 hr and lipids were extracted either immediately afterwards or 24 hr post exposure. The samples were then analyzed by LC/MS/MS and the levels of \( \beta \)-epoxide were quantitated. As shown in figure 3, \( \beta \)-epoxide was virtually undetected in cells exposed to ambient air, but there was a dose dependent formation of \( \beta \)-epoxide in the ozone exposed cells. The cellular levels of \( \beta \)-epoxide at 24 hr post ozone exposure were significantly decreased, suggesting that \( \beta \)-epoxide was either unstable in this environment, or that it was being metabolized by the 16-HBE cells.

**5\( \beta \),6\( \beta \)-Epoxycholesterol metabolism.** The fate of \( \beta \)-epoxide was further examined in cultured 16-HBE cells. Cells (1 x 10^5) in 3 ml media were treated with 1 \( \mu \)M \( \beta \)-epoxide, and samples were taken at various time points for analysis of the remaining quantity of \( \beta \)-epoxide in the lipid extract. The epoxide concentration was found to decrease over time with an apparent half-life of approximately 10 hr (data not shown). In media alone over the same time period, there was no decrease in epoxide concentration suggesting that this compound was stable in this aqueous
environment.

Previous studies have suggested that the major metabolite of β-epoxide was cholestane-3β,5α,6β-triol (3,5,6-triol) due to the action of cholesterol epoxide hydrolase (20,28). The 3,5,6-triol was synthesized (24) and the HPLC and mass spectrometric characteristics of this metabolite were studied. A sensitive and specific LC/MS/MS assay was developed using the mass transition, m/z 438 → 385 to detect the elution of 3,5,6-triol with a retention time of 8 minutes. The 3,5,6-triol was observed when 16-HBE cells (1 × 10⁶) were incubated with 3 μM β-epoxide (Figure 4A), and the quantity did increase with time of incubation. However, after 24 hr of incubation, the low levels of this metabolite did not correspond with the marked decrease in β-epoxide over this time period, suggesting another metabolite might in fact be formed. A new metabolite was observed with an [M+NH₄]⁺ ion at m/z 436 that could be collisionally activated to yield a product ion at m/z 383. This same mass transition had been previously used to detect an expected ozonolysis product, namely 3β-hydroxy-5-oxo-5,6-secocholestane-6-al (5,6-secocholesterol). However, this 5,6-secocholesterol had a retention time of 8 min and was not detected in the media of cells treated with β-epoxide. An abundant component with a retention time of 10 minutes was detected by this ion transition, as an unexpected metabolite of β-epoxide (Figure 4B). This unknown metabolite was found to substantially increase with further cell incubation (data not shown). Unmetabolized β-epoxide was still present in the media as indicated by Figure 4C. The elution of an internal standard (deuterated analog of β-epoxide) added during sample work-up was used to precisely mark the elution of β-epoxide (Figure 4D).

In order to obtain sufficient quantities of the unknown metabolite for further structural studies, lipids were extracted from 16-HBE cells (10⁷) after treatment with 10 μM β-epoxide for 24
hr. The samples were then pooled and purified by reversed phase HPLC. A small portion of the HPLC effluent was monitored by MS/MS for the transition of the metabolite, and the fraction containing this metabolite was collected. Collisional activation of the [M+NH₄]⁺ ion formed during electrospray ionization of this fraction yielded a product ion mass spectrum that was identical to the collisional activation of 5,6-secosterol ([M+NH₄]⁺ at m/z 436) and provided little unique structural information. The collision induced dissociation of the ion derived from this metabolite yielded an ion corresponding to the loss of NH₃ (m/z 419) and three additional ions corresponding to the subsequent losses of each oxygen atom as water (m/z 401, 383, and 365). Analysis of the unknown metabolite by electron ionization mass spectrometry required derivatization to the trimethylsilyl ether in order to facilitate gas chromatographic separation (Figure 5a). A molecular ion at m/z 562 (M⁺) was observed which corresponded to the addition of 144 daltons to the expected molecular weight of the metabolite, indicating the addition of two trimethylsilyl groups and thus the presence of two hydroxyl groups in the structure. The expected third oxygen atom was not derivatized, suggesting that it was either an oxo or an epoxide moiety, which would account for the single unsaturation relative to 3,5,6-triol.

Treatment with perchloric acid opened both β-epoxide and α-epoxide to vicinal diols; however the unknown metabolite was resistant to perchloric acid treatment, suggesting that an epoxide was not present. Sodium borohydride reduction of the metabolite yielded a product with a retention time 8 min. Electrospray ionization mass spectrometry of this reduced product gave an abundant [M+NH₄]⁺ ion, m/z 438, that was collisionally activated to yield losses of three water molecules and ammonia (m/z 420, 403, 385, 367). This mass spectrum and retention time was identical to 3,5,6-triol suggesting that the unknown metabolite contained a reducible ketone or...
aldehyde in place of one of the hydroxyl groups. In separate experiments, treatment of \( \alpha \)-epoxide and \( \beta \)-epoxide with sodium borohydride did not alter these structures, as revealed by electrospray mass spectra and HPLC retention times that were identical to the starting material.

When cells were treated with \( \mathrm{d}_6 \)-5\( \beta \),6\( \beta \)-epoxycholesterol, a deuterated form of the unknown metabolite was formed that retained only five of the original deuterium atoms, as observed by electron ionization mass spectrometry of the bis-trimethylsilyl derivative (\( \text{M}^+ \), m/z 567). Since the \( \mathrm{d}_6 \)-5\( \beta \),6\( \beta \)-epoxycholesterol was labeled at carbon atoms 2, 2\', 3, 4, 4\', and 6, this suggested that the oxo group was most likely at either carbon-3 or carbon-6 of the original cholesterol structure as these were the sites of a single deuterium atom label. Analysis of the TMS derivative by GC/MS gave a characteristic steroidal fragment ion at m/z 129. This ion was shifted in the spectrum of the TMS derivative of the deuterated metabolite by two mass units to m/z 131, consistent with this ion being derived from the carbon-3 TMS ether substituent (29). Therefore, the hydroxyl group at carbon-3 was likely intact, suggesting that the substitution of oxygen as an oxo group was in fact at carbon-6. All data was consistent with the unknown metabolite as cholestan-6-oxo-3,5-diol. This compound was synthesized (25) and was found to have the same retention time and electron ionization mass spectrum as the unknown metabolite (Figure 5b).

**Biological activity of 5\( \beta \),6\( \beta \)-epoxycholesterol and cholestan-6-oxo-3,5-diol.** Previous investigators studied the cytotoxic effects of cholesterol epoxides in Chinese hamster lung fibroblasts, rabbit aortic endothelial cells and in the human monocytic cell line, U937. In these systems \( \beta \)-epoxide was more cytotoxic than the \( \alpha \)-epoxide (20,30,31). Additionally, the \( \beta \)-epoxide was shown to be more mutagenic than the \( \alpha \)-epoxide in mouse embryo cells (32). Similar isomer specificity was observed during incubation of 16-HBE cells with the epoxides. The \( \alpha \)-epoxide did not cause cell death even at levels of 30 \( \mu \)M, while the \( \beta \)-epoxide was found to cause significant
cytotoxicity in a dose and time dependent manner (Figure 6). The highest concentration used, 30 μM, led to significant cytotoxicity by 24 hr; however the 10 μM dose did not have an effect until 48 hr of incubation when over 50% of the cells incorporated the trypan blue dye. The cumulative effect of β-epoxide in causing cell death suggested that the oxysterol was altering or impairing some biochemical pathway rather than acting via a receptor mediated event.

Early studies of related oxysterols suggested a mechanism of cell death related to the inhibition cholesterol synthesis (23,33), therefore the ability of β-epoxide to impair cholesterol biosynthesis was examined. After a 24 hr treatment of 16-HBE with β-epoxide, radiolabeled [14C]-acetate was incubated with the cells for 3 hr. The neutral lipids were extracted and separated by TLC. The ratio of radioactivity present in cholesterol to that present in triacylglycerol was determined and compared to untreated cells as a measure of cholesterol synthesis inhibition (23). The values obtained from a representative experiment are shown in Table 1. Cholesterol synthesis was found to be inhibited even with nanomolar concentrations of the epoxide, and was almost completely inhibited at concentrations that were found to cause significant cytotoxicity (Figure 7). Both β-epoxide and 6-oxo-3,5-diol were able to inhibit cholesterol synthesis using this assay with an IC$_{50}$ of 350 nM. The effect was more potent in the cultured lung cell line A549, with an IC$_{50}$ of 150 nM (data not shown). The role of cholesterol depletion in the observed cytotoxicity was examined by attempting to rescue the cells by supplementation of the media with cholesterol. Cells were protected from the effects of the oxysterol 6-oxo-3,5-diol by the addition of cholesterol in a dose dependent manner (Table 1), as exemplified when cells treated with 15 μM 3,5-diol-6-one that would have resulted in approximately 50% cell death had less than 20% cell death when 30 μM cholesterol was coincubated with the oxysterol.
DISCUSSION

Formation of 5β, 6β-epoxycholesterol has been previously observed as a result of cholesterol peroxidation and autooxidation (34). This oxysterol was shown to initiate cellular apoptosis in some systems (20, 31) and to play a role in lipid loading of macrophages (35), but had not previously been described as a major product of cholesterol ozonolysis in lipid membranes. Ozonolysis of olefins is thought to characteristically proceed via formation of a Criegee ozonide which breaks the carbon-carbon double bond leaving a keto or aldehyde moiety and a hydroperoxy hydroxyl acetal substituent at these carbons atoms. The reaction of ozone with cholesterol by this mechanism would be expected to yield 3β-hydroxy-5-oxo-5,6-secocholestan-6-al. An alternative, less appreciated mechanism of ozone involves addition of one oxygen atom of the trioxygen molecule to a double bond, followed by loss of diatomic oxygen, which results in epoxidation but not scission of the carbon-carbon bond (36). The yield of such epoxides during ozonolysis can be dependent on the solvent used and the degree of substitution at the target double bond. For example, lanosterol derivatives have been shown to primarily form epoxides at the very hindered 8,9 double bond during ozonolysis in methylene chloride (37). Alternatively, β-epoxide could form via lipid peroxidation since it has been proposed that ozone can initiate the formation of radical species (38). However, other studies of cholesterol ozonolysis in solution suggest that β-epoxide is a unique cholesterol ozonolysis product rather than a secondary result of peroxide formation (11).

The identification of 6-oxo-3,5-diol as the major metabolite of β-epoxide that accumulated in 16-HBE cells was not anticipated, since other studies had identified 3,5,6-triol as the major metabolite of both α-epoxide and β-epoxide (20, 28). A metabolism study of orally administered 3,5,6-triol in rat yielded 6-oxo-3,5-diol as a major metabolite (39). In fact, treatment of 16-HBE cells with 3,5,6-triol led to the production of 6-oxo-3,5-diol suggesting that 3,5,6-triol was an
intermediate formed during the metabolism of $\beta$-epoxide to 6-oxo-3,5-diol (data not shown). Very little is known about the biological activity of 6-oxo-3,5-diol; however, there have been a few studies suggesting it may be an endogenous ligand for cytosolic-nuclear tumor promoter binding protein, with which PMA had been shown to bind with high affinity (40,41). Furthermore, 6-oxo-3,5-diol has been used for photoaffinity labeling of this protein (42). The observation of 6-oxo-3,5-diol as a major metabolite of $\beta$-epoxide is significant aside from its potential biological activity, in that this compound is isobaric with other cholesterol products suggested as biomarkers for ozone exposure, such as 5,6-secosterol and its corresponding aldol condensation product (molecular weight of 418 daltons). Careful analysis of the cholesterol derived products formed during ozone exposure, beyond just molecular weight determination, would be required to distinguish between these compounds.

There is reasonable debate about the claims that oxysterols are cytotoxic since levels used for *in vitro* studies are extraordinarily high, most often in the low micromolar range. Our study also found these levels necessary for cytotoxicity; however, it is reasonable that these levels could be achieved *in vivo* after exposure to ozone. Kafoury et al. (7) treated human bronchial epithelial cells, BEAS-2B, with micromolar concentrations of an ozonized phosphatidylcholine lipid product, and calculated the amount of lipid which partitioned into the cellular membrane. The micromolar concentration in media translated to low nanomolar concentrations in the cellular membrane, supporting the use of these high levels for *in vitro* studies. Direct exposure of cells to ozone led to accumulation of nanomolar concentrations of $\beta$-epoxide in the cellular membranes suggesting that these levels may be achieved in the lung in areas where the surfactant lining layer is sufficiently thin. Furthermore, the biologically active concentrations required for inhibition of cholesterol biosynthesis are far lower than those required for cytotoxicity, and could be readily achieved during
in vivo exposure of ozone. Both β-epoxide and 6-oxo-3,5-diol were shown to be potent inhibitors of cholesterol synthesis, with IC₅₀ values of 350 nM.

Synthesis of cholesterol from acetate involves numerous steps, and several of the intermediates are involved in other biochemical pathways (43). For instance, prenylation of proteins requires transfer of farnesyl and geranylgeranyl isoprenoid pyrophosphates (which are intermediates in cholesterol synthesis) to cysteine residues at the C-terminus of a target proteins (44). Many of the proteins that are modified by this process are small guanosine triphosphate binding proteins involved in cell cycle regulation, such as Ras and the Rho family of proteins (45). Inhibitors of cholesterol synthesis have been shown to interfere with the lipid modification of these proteins leading to changes in cell signaling and suggesting that this pathway is involved in the observed cytotoxicity of statins and phytosterols (45, 46). The oxysterols formed during ozone exposure in the lung may also cause changes in the prenylation of proteins due to their effects on isoprenoid synthesis. Some studies suggest that blocking protein prenylation can cause changes in inflammatory signaling (47,48). Treatment of macrophages with lovastatin was shown to stimulate low levels of TNF-α and to enhance LPS stimulated TNF-α production (47). Also, mevalostatin and lovastatin were shown to upregulate cyclooxygenase-2 expression in human aortic smooth muscle cells (48). Oxysterols may similarly act to enhance the inflammatory response observed during ozone exposure in the lung.

In conclusion, the observation of biologically active ozonized cholesterol products formed during exposure of pulmonary surfactant and lung epithelial cells to ozone supports the theory that ozonized lipids and particularly oxysterols may mediate the toxicity of ozone. The two compounds seen in this study, β-epoxide and 6-oxo-3,5-diol, have not been studied extensively, but similar
oxysterols, such as 7β-hydroxycholesterol, 25-hydroxycholesterol, and 7-ketocholesterol, have been implicated in the initiation of inflammatory signaling associated with atherosclerosis (49-51). The formation of β-epoxide and 6-oxo-3,5-diol in the lung may also play a role in the increased risk of cardiopulmonary diseases observed with high ozone exposure in epidemiological studies (52,53). Finally, with regard to the recent interest in ozone as a biochemical product during inflammation (54), the biological role of β-epoxide and its metabolites needs to be examined as they may be formed in cellular membranes by reaction with ozone formed in vivo, and these compounds may play a significant role in the formation of lipid loaded macrophages and the development of atherosclerosis.
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FIGURE LEGENDS

Figure 1  Radiochromatogram of cholesterol ozonolysis products extracted from rat lung surfactant exposed to 2 ppm ozone for 4 hr. Rat lung surfactant was spiked with $^{14}$C cholesterol and chromatographed by RP-HPLC with on-line mass spectrometry using both positive and negative ion electrospray in separate experiments. The component eluting at 16.5 minutes (A) had an abundant negative ion observed at m/z 511, corresponding to the [M+OAc]$^-$ ion derived from 5-hydroperoxy-B-homo-6-oxa-cholestane-3β,7a-diol (Scheme 1). The component eluting at 22 minutes (B) yielded an abundant positive ion at m/z 420. Coelution with a synthetic standard revealed this to be an [M+NH$_4$]$^+$ ion derived from 5β,6β-epoxycholesterol (see text).

Figure 2  5,6-Cholesterol epoxide formation in rat surfactant exposed to ozone. Rat surfactant was diluted to a concentration of 30 μg cholesterol/ml, and 100 μl aliquots were ozonized in 35 mm wells for 4 hour at concentrations of 0.25 ppm, 0.5 ppm, and 1.0 ppm or exposed to filtered air. Neutral lipids were extracted and quantitated using stable isotope dilution mass spectrometry. Low to undetectable levels of both of β-epoxide (black bars) and α-epoxide (striped bars) were observed in the filtered air controls. Nanogram levels of both epoxide isomers were observed in the ozone treated samples (n=3).

Figure 3  Formation of 5β,6β-cholesterol epoxide in 16-HBE cells exposed to ozone. Approximately 1 x 10$^6$ cells (16-HBE) were exposed to indicated levels of ozone for 1 hour. Analysis of the samples was carried out immediately following ozone exposure (grey bars), or after 24 hour incubation at 37°C (striped bars). During analysis, aliquots of the cells were counted using a hemocytometer, and then the lipids were extracted for quantitation using stable isotope mass
spectrometry. The amount of β-epoxide formed was normalized to the number of cells in each sample and is shown as ng/100,000 cells (n=3).

**Figure 4** Representative reverse phase HPLC separation of the metabolites formed after 24 hr incubation of β-epoxide with 16-HBE cells. Multiple reaction monitoring (LC/MS/MS) was used to detect specific products based on mass transition and retention time. The chromatogram shows the relative intensity of the peaks for (A) the known metabolite, 3,5,6-triol (m/z 438 → 385) and (B) the unknown metabolite (m/z 436 → 383). The known ozonized cholesterol product 5,6-secocholesterol has the same transition (m/z 436 → 383), but a retention time of 7.5 min. The peak corresponding to the unmetabolized (C) β-epoxide (m/z 420 → 385) and (D) its deuterated isotope (m/z 426 → 391) are also shown.

**Figure 5** Electron ionization (70 eV) mass spectrum of the TMS derivative of A.) unknown metabolite and B.) synthesized cholestan-6-oxo-3,5-diol. The structures of indicated ions are tentatively suggested based on the shifts in mass to charge ratios observed in both d5-TMS and d5-cholestane-6-oxo-3,5-diol.

**Figure 6** Effect of time and dose of 5β,6β-epoxycholesterol on the viability of 16-HBE cells as measured by trypan blue exclusion. Cells (2 x 10^5) were treated with indicated concentrations of β-epoxide for different lengths of time or with 30 μM α-epoxide for 40 hr. At least 100 cells were counted for each representative point.
Figure 7  Inhibition of cholesterol synthesis by β-epoxide and 6-oxo-3,5-diol. After treatment of 16-HBE cells (1 x 10⁶) with β-epoxide or 6-oxo-3,5-diol for 24 hr, the cells were incubated with [1-¹⁴C] acetate for 3 hr for incorporation of radiolabel into newly synthesized lipids. The neutral lipids were extracted from the sample and analyzed by thin layer radiochromatography. Percent inhibition of cholesterol synthesis was measured by comparison of the newly synthesized cholesterol to triacylglycerol ratio in untreated control cells and oxysterol treated cells. A logarithmic dose response curve was observed (n=2).
Table 1  Representative data of the effect of 5β,6β-epoxycholesterol and cholestan-6-oxo-3,5-diol on cholesterol biosynthesis and cell viability in 16-HBE cells. Cells (approximately 1 x 10^5 in 35 mm wells) were treated with the indicated concentrations of β-epoxide. After 24 hr, \(^{14}\)C-acetate (7 µCi per dish) was added and incubation carried out for an additional 3 hr. Suppression of cholesterol biosynthesis by β-epoxide at the indicated concentrations was measured by comparing the ratio of \(^{14}\)C-acetate incorporation into cholesterol (cpm) with that of triacylglycerol (cpm). Comparison of the observed ratio from controls (untreated) to that observed for β-epoxide exposed cells was used to calculate the percent inhibition of cholesterol synthesis. Similar data for 6-oxo-3,5-diol were obtained (Figure 7). Cells could be rescued from the cytotoxic effects of the 6-oxo-3,5-diol (or β-epoxide, not shown) by addition of cholesterol to the media before the addition of oxysterol to cells. Incubation with exogenous cholesterol during the 48 hr oxysterol treatment gave a dose dependent protection from the cytotoxic effects of 15 µM 6-oxo-3,5-diol as measured by trypan blue exclusion.

| µM beta epoxide | Cholesterol (cpm) | Triacylglycerol (cpm) | ratio  |
|-----------------|-------------------|----------------------|--------|
| control         | 985.3             | 420.3                | 2.344  |
| 0.03            | 567.8             | 367.3                | 1.546  |
| 0.1             | 724.5             | 397.5                | 1.823  |
| 0.3             | 302.7             | 296.5                | 1.021  |
| 1               | 221.5             | 372.6                | 0.594  |
| 3               | 182.5             | 440.4                | 0.414  |
| 10              | 97.3              | 197.6                | 0.492  |

| µM 3,5-diol-6-one | µM cholesterol | Percent Cell Death | S.E.  |
|------------------|----------------|--------------------|-------|
| 0                | 0              | 9.5                | ± 2.7 |
| 15               | 0              | 47.8               | ± 8.0 |
| 15               | 1              | 41.6               | ± 1.5 |
| 15               | 3              | 31.2               | ± 3.1 |
| 15               | 10             | 29.2               | ± 4.3 |
| 15               | 30             | 16.3               | ± 4.3 |
Scheme 1

5-hydroperoxy-6-homo-6-oxa-cholest-3β,7α-diol

5,6-secosterol

β-epoxide

α-epoxide

3,5,6-triol

6-oxo-3,5-diol
Figure 3

- **0 hr**
- **24 hr**

**ng β-epoxide/100,000 cells**

- **0 ppm**
- **0.2 ppm**
- **0.5 ppm**
- **1.0 ppm**
Figure 4

A

m/z 438 → 385

B

m/z 436 → 383

C

m/z 420 → 385

D

m/z 426 → 391
Figure 5

A.

B.
Figure 6

% cell death vs. hours

- Control
- 10 μM β-epoxide
- 20 μM β-epoxide
- 30 μM β-epoxide

30 μM α-epoxide
Figure 7

Inhibition of Cholesterol Synthesis

% Cholesterol Synthesis Inhibition (relative to control cells)

Cholesterol Concentration (µM)

- β-epoxide
- 6-oxo-3,5-diol
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Melissa K. Pulfer and Robert C. Murphy

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