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Ozone Exposure in Vivo and Formation of Biologically Active Oxysterols in the Lung

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ABSTRACT

Ozone toxicity in the lung is thought to be mediated by products derived from the reaction of ozone with components of the lung epithelial lining fluid. Cholesterol is an abundant component of this epithelial lining fluid, and it is susceptible to ozonolysis, yielding several stable products including 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and 5β,6β-epoxycholesterol. Both 5β,6β-epoxycholesterol and its metabolite, cholestan-6-oxo-3,5-diol, have been shown to cause cytotoxicity in vitro, suggesting that they may be potential mediators of ozone toxicity in vivo. An ozone-sensitive mouse strain, C57BL/6J, was exposed to varying concentrations of ozone (0.5–3.0 ppm), and subsequently the levels of these cholesterol ozonolysis products were quantitated by electrospray ionization mass spectrometry in bronchoalveolar lavage fluid, lavaged cells, and lung homogenate. An ozone dose-dependent formation of these biologically active oxysterols was observed in vivo, supporting a role for these compounds in ozone toxicity. Since the 5β,6β-epoxycholesterol metabolite, cholestan-6-oxo-3,5-diol, was isobaric with other cholesterol ozonolysis products, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and its aldol condensation product, 3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde, detailed mass spectral analysis using electron impact ionization was utilized to differentiate these isobaric cholesterol ozonolysis products. The specific detection of cholestan-6-oxo-3,5-diol in lung homogenate after ozone exposure established formation of 5β,6β-epoxycholesterol within the lung after exposure to 0.5 ppm ozone.

Ozone is a major air pollutant that has been known to cause toxic pulmonary effects in animals and man for decades (Stokinger, 1965). Various adverse sequelae of ozone exposure have been documented including increased airway hyperresponsiveness, epithelial sloughing, and neutrophil influx in the airways (Schelegle et al., 1991; Hyde et al., 1992; Park et al., 2004a). The primary mechanism for this observed ozone toxicity has not been defined. Calculations suggest that the high reactivity of ozone and its low solubility in water would prevent it from passing through the lung epithelial lining fluid to act directly with the underlying epithelial cells (Pryor, 1992). Lung epithelial lining fluid, which contains pulmonary surfactant, is composed of almost 95% lipids (Sadana et al., 1988; Hall et al., 1994); thus, it has been proposed that ozone exerts its toxic effects via a lipid mediator, which is formed during the interaction of ozone with lipids in the pulmonary surfactant (Pryor et al., 1995; Postlethwait et al., 1998). Various studies have shown that oxidized lipids can act as signaling molecules (Kafoury et al., 1998; Uhlson et al., 2002); for example, lysophospholipids, which could potentially be formed during ozonolysis, have been shown to initiate PAF-like activity (Marathe et al., 1999).

Cholesterol, which is the most abundant neutral lipid in human pulmonary surfactant (Sadana et al., 1988), has a double bond that is susceptible to attack by ozone (Bailey, 1957). Multiple products have been described to form during the reaction of ozone with cholesterol and the product yields have been shown to depend on ozonolysis conditions (Gumulka and Smith, 1983; Jaworski and Smith, 1988). Recently, our laboratory found that 5β,6β-epoxycholesterol (β-epoxide) (Scheme 1) was a major product of cholesterol ozonolysis in a lipid environment (Pulfer and Murphy, 2004). This oxysterol has been studied as a product of cholesterol

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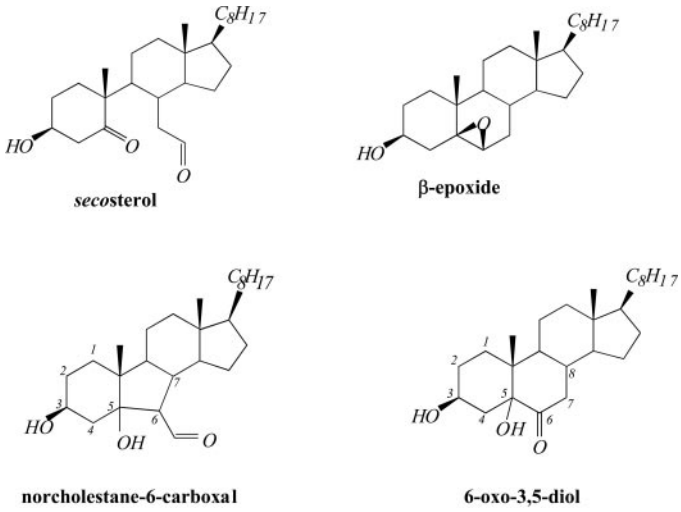
ABBREVIATIONS: β-epoxide, 5β,6β-epoxycholesterol; 6-oxo-3,5-diol, cholestan-6-oxo-3,5-diol; *seco*sterol, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; norcholestane-6-carboxal, 3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde; BSTFA, bis(trimethylsilyl)fluoroacetamide; RP-HPLC, reversed phase high-pressure liquid chromatography; MRM, multiple reaction monitoring; HBSS, Hanks' balanced salt solution; BAL, bronchoalveolar lavage; GC, gas chromatography; MS, mass spectrometry; amu, atomic mass unit(s); TMS, trimethylsilyl; OTMS, octadecyltrimethoxysilane.



Scheme 1.

autooxidation and lipid peroxidation (Sevanian and McLeod, 1987) and has been shown to cause cytotoxicity in in vitro systems (Sevanian et al., 1991; O'Callaghan et al., 2001). Recent studies have shown that a major metabolite of β-epoxide in cultured human bronchial epithelial cells, cholestan-6-oxo-3,5-diol (6-oxo-3,5-diol), is also cytotoxic (Pulfer and Murphy, 2004). Therefore, formation of β-epoxide and 6-oxo-3,5-diol in lung surfactant during ozone exposure could play a role in the lung epithelial cell necrosis and sloughing observed soon after ozone exposure (Hyde et al., 1992).

The formation of cholesterol-derived products has been studied after ozone exposure in animals (Pryor et al., 1992). The products monitored were 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (*seco*sterol) and its aldol condensation product, 3β-hydroxy-5β-hydroxy-B-norcholestan-6β-carboxaldehyde (norcholestane-6-carboxal) (Scheme 1), and the method of detection for these products involved only molecular weight analysis of the dinitrophenylhydrazone derivatives. Formation of cholesterol epoxides has not been studied in relationship to ozone exposure; however, cholesterol epoxide formation in the lung was previously studied after exposure of rats to high levels of NO_2, which induces lipid peroxidation (Sevanian et al., 1979). A modest increase in the levels of epoxide was seen after exposure to this potent oxidant, suggesting that cholesterol in pulmonary surfactant is susceptible to oxidation. To further assess the effect of ozone on cholesterol oxidation, we used an in vivo model of acute ozone exposure (Park et al., 2004a,b) to study the formation of β-epoxide and its cellular metabolite, 6-oxo-3,5-diol. In the present study, we describe the formation of these cholesterol oxidation products in the lungs of mice exposed to ozone concentrations as low as 0.5 ppm.

Materials and Methods

Materials. Cholesterol and bis(trimethylsilyl)fluoroacetamide (BSTFA) were purchased from Sigma-Aldrich (St. Louis, MO). Stable isotope-labeled 2,2′,3,4,4′,6-d_6-cholesterol (98%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Quantitation of Epoxycholesterol and 6-Oxo-3,5-diol. The alpha and beta isomers of 5,6-epoxycholesterol were synthesized as previously described (Pulfer and Murphy, 2004). For reversed phase high-pressure liquid chromatography (RP-HPLC) solvents used were solvent A, methanol/water/acetonitrile (v/v/v; 60:20:20) with 1 mM ammonium acetate and solvent B, methanol with 1 mM ammonium acetate. Quantitation was carried out using stable isotope dilution mass spectrometry. Samples were introduced onto the mass spectrometer using a 150- × 1.0-mm Columbus C18 column (Phenomenex, Torrance, CA). The flow rate was 50 μl/min, and the gradient increased from 75% to 100% B over 10 min followed by 100% B for 20 min. Multiple reaction monitoring (MRM) analysis was carried out on a Sciex API-2000 mass spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA). The oxidized cholesterol products formed $[M+NH_4]^+$ ions in the positive ion mode with an ion spray voltage of 4500 V, declustering potential of 40 V, focusing potential of 350 V, and collision energy of 12 V. Nitrogen was used in the collision cell with a collision gas thickness of 2.17×10^{15} mol/cm^2. The transitions monitored were *m/z* 420 → 385 for the 5,6-epoxycholesterol isomers, *m/z* 426 → 391 for d_6-5β,6β-epoxycholesterol, *m/z* 404 → 369 for cholesterol, and *m/z* 436 → 383 for 6-oxo-3,5-diol, *seco*sterol, and norcholestane-6-carboxal. The dwell time for each transition was 800 ms. The standard curve was linear for a range of 0.31 to 320 ng for β-epoxide and 6-oxo-3,5-diol. The limit of detection was defined as 0.16 ng, which yielded a peak that was 4-fold greater than background noise. Therefore, for points that were lower than the limits of detection, a value of <0.16 ng was assigned to the sample.

Animals. Eight- to 12-week-old female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and housed under pathogen-free conditions and maintained in the Biological Research Center at the National Jewish Medical and Research Center. All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Ozone Exposure. Mice were exposed to different concentrations (0.5–3.0 ppm) of O_3 for 3 h ($n = 4$). They were placed in stainless steel wire cages set inside 240-liter laminar flow inhalation chambers. HEPA-filtered room air was passed through these chambers at 25 changes/h. Room temperature was maintained at 20 to 25°C. Ozone was generated by directing compressed medical-grade oxygen through an electrical discharge O_3 generator (Sander Ozonizer, model 25, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) located upstream of the exposure chamber. The O_3-air mixture was metered into the inlet air stream with mass flow controllers (MKS Instruments Inc., Model #1359C, Andover, MA). Simultaneous exposure to HEPA-filtered air was carried out in a separate chamber with age- and treatment-matched control animals. Ozone concentrations were continuously monitored at mouse nose levels within the chamber with a photometric O_3 analyzer (model 400A; Advanced Pollution Instrumentation, Inc., San Diego, CA) and recorded on a strip-chart recorder. Calibration of the O_3 analyzer was performed by the Colorado Department of Public Health and Environment.

Bronchoalveolar Lavage and Lung Preparation. Mice were euthanized 6 h after the ozone exposure, and the lungs were lavaged via the tracheal tube with 1 ml of Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). The bronchoalveolar lavage fluid (BAL) was centrifuged at 2000 rpm for 10 min, and the supernatant was transferred to glass tubes. The cell pellet was resuspended in 1 ml of HBSS and subjected to a second round of centrifugation. The supernatant was combined with that from the first centrifugation, and the cell pellet was resuspended in 200 μl of HBSS. Deuterated β-epoxide (40 ng in 25 μl) was added to the samples of cell-free lavage fluid and cells. A 10-μl aliquot of the cell suspension was counted using a hemocytometer for total cell count, excluding red blood cells. Whole lungs were dissected from the mice, and 400 ng of internal standard in 25 μl ethanol was added prior to homogenation in HBSS.

Lung homogenate, lavaged cells, and cell free lavage supernatants were diluted to a final volume of 1 ml with water. Neutral lipid extraction was achieved by the addition of 2 ml of methanol and 3 ml of iso-octane, and the organic phase was dried under nitrogen. The

lipid extract from lung homogenate was resuspended in 2 ml of ethanol, and a 40-μl aliquot was diluted with 40 μl of solvent A for mass spectral analysis. Total extracts from the lavage fluid and cells were resuspended in 80 μl of solvent A for analysis. Samples were quantitated by MRM as described above.

Synthesis of Cholestan-6-oxo-3,5-diol and 3β-Hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde and Purification of in Vivo Unknown. Cholestan-6-oxo-3,5-diol was synthesized as previously described (Pulfer and Murphy, 2004). Briefly, cholestanetriol was synthesized by opening the epoxide moiety of β-epoxide (10 mg) to a vicinal diol by treatment with 0.5 ml of perchloric acid in 4 ml of tetrahydrofuran/H_2O/acetone (v/v/v; 4:1:0.5). The resulting cholestanetriol was extracted in dichloromethane and purified by RP-HPLC using the conditions described above. Cholestanetriol (10 mg) was dissolved in 4.5 ml of ether, 750 μl of methanol, and 750 μl of water and stirred with *N*-bromosuccinimide (108 mg) for 3 h at room temperature to yield the product, 6-oxo-3,5-diol. The reaction was diluted with water and extracted with dichloromethane. Purification was achieved by RP-HPLC.

The cholesterol ozonolysis product, norcholestane-6-carboxal, was synthesized by modification of published methods (Gumulka and Smith, 1983; Miyamoto et al., 2001; Wentworth et al., 2003). Cholesterol (5 mg) was suspended in 2 ml of tetrahydrofuran/H_2O (1:1; v/v) and exposed for 3 min to ozone generated by passing oxygen at 1 ml/min through a tesla coil (Supelco, Bellefonte, PA) fitted with a glass sleeve covering the high-voltage electrode. Lipids were then extracted from the solution with methylene chloride and dried under nitrogen. The primary ozonide was resuspended in 2.5 ml of acetic acid/H_2O (9:1; v/v) and reduced by stirring with 32.5 mg of zinc dust for 3 h at room temperature. The reaction mixture was washed with water (5 $\times$ 4 ml), and the resulting *seco*sterol was purified by RP-HPLC as described above. Purified *seco*sterol (5 mg) in benzene (2.5 ml) was stirred overnight with excess activated alumina (2 h at 110°C). Benzene was evaporated under nitrogen and lipids extracted using the method of Bligh and Dyer (1959). The lipid extract was dried under nitrogen, and norcholestane-6-carboxal was purified by RP-HPLC.

The total neutral lipid extract from the lung homogenate of three mice lungs exposed to 3 ppm ozone for 3 h was pooled and dried under vacuum. The sample was resuspended in 100 μl of solvent A and chromatographed by RP-HPLC on an Ultramex 5 C18 (250 $\times$ 4.6 mm) reverse phase column (Phenomenex) with a flow rate of 1 ml/min using the gradient listed above. A small fraction of the effluent (20 μl/min) was split to the mass spectrometer for on-line MRM analysis of the mass transition 436 $\rightarrow$ 383. The remaining effluent was collected in 1-min fractions, and the fractions corresponding to the aforementioned mass transition were pooled and dried under vacuum.

NMR analysis of the synthesized norcholestane-6-carboxal was kindly provided by Dr. David Jones (University of Colorado Health Sciences Center). Samples were dissolved in deuterated chloroform for proton NMR analysis (100 atom % D; Aldrich Chemical Co., Milwaukee, WI). Spectra were recorded on a Varian Inova Spectrometer with a proton frequency of 500 MHz. COSY and TOCSY proton assignments for the A and B rings of the synthesized compound were consistent with the norcholestane-6-carboxal structure: ^{1}H NMR ($CDCl_3$) δ 9.721 (d, CHO), 4.141 (m, H-3), 2.256 (m, H-4a), 2.349 (m, H-6), 2.214 (m, H-7), 1.837 (m, H-4b), 1.763 (m, H-2a), 1.724 (m, H-2b), and 1.483 (m, H-1).

Gas Chromatography (GC)/Mass Spectrometry (MS). For analysis of standards, 5 μg of sample was dried under vacuum. Samples were derivatized by the addition of 50 μl of BSTFA and 50 μl of acetonitrile followed by heating to 65°C, yielding the trimethylsilyl (TMS) derivatives. For the unknown in vivo sample (molecular mass, 418 amu), a small aliquot (5%) was dried under vacuum and similarly derivatized with BSTFA. An aliquot (2 μl) 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 to 260°C at 20°C/min and 260 to 310°C at 4°C/min on a 30 m ZB-1 column (Phenomenex) with a 0.25-mM i.d. and a 0.25-μm stationary film thickness.

Data Analysis. Statistical calculations were performed using Excel (Microsoft, Redmond, WA). Data are presented as mean $\pm$ S.E.M. For analysis of significance, the data were analyzed by Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

The mouse strain C57BL/6J was chosen for analysis of β-epoxide and 6-oxo-3,5-diol in vivo because of the unique susceptibility of this mouse strain to ozone toxicity (Kleeberger et al., 1997). Quantitation of cholesterol ozonides in mice after ozone exposure was performed by stable isotope dilution mass spectrometry. Representative MRM traces from the BAL fluid of a control mouse and an ozone-treated mouse are shown in Fig. 1. Relative to added internal standard, greater ion intensities were observed for the peaks corresponding to cholesterol ozonide products in the ozone-treated mouse BAL than in the control mouse BAL.

Ozone Dose-Dependent Formation of Oxysterols in Vivo. Mice were treated with room air or 3 ppm ozone for 3 h, and at various time points after exposure, the cholesterol ozonide products were quantitated in lung lavaged fluid (supernatant), lavaged cells, and lung homogenate. The concentration of both β-epoxide and its metabolite, 6-oxo-3,5-diol, were elevated above filtered air-treated mice for 24 h after exposure, with the highest levels found at 6 h postexposure (data not shown). The 6-h time point was therefore chosen for the analysis of BAL fluid and whole-lung homogenate from mice exposed to increasing concentrations of ozone (0.5, 1.0, 2.0, or 3.0 ppm). Quantitative results for cellular lipids were normalized to the number of cells in the lavage sample and reported as nanograms per 10^5 cells. A trend for increased β-epoxide was seen in the lipid extract from lavaged cells over the ozone dose range studied (Fig. 2A). The lipid extract from the lavage supernatant also showed a trend for increased β-epoxide, and levels were significantly different from control mice at all ozone exposure doses (Fig. 2B; $p < 0.05$, $n = 4$). A dose response was not observed for increased β-epoxide in lung homogenates from ozonized mice (Fig. 2C).

The levels of the β-epoxide metabolite, 6-oxo-3,5-diol, in bronchoalveolar lavaged cells of control C57BL/6J mice were below the limits of detection (<0.16 ng). However, in mice exposed to 3.0 ppm ozone, 16.8 ng of 6-oxo-3,5-diol per 10^5 cells was detected (Fig. 3A). Quantitation of 6-oxo-3,5-diol in the lavage supernatants revealed significant increases over control mice for all doses of ozone studied (Fig. 3B; $p < 0.05$; $n = 4$). In remarkable contrast to β-epoxide, there was a dose-dependent increase in the formation of 6-oxo-3,5-diol in lung homogenates (Fig. 3C).

Trypan blue exclusion analysis of cells collected from bronchoalveolar lavage (with the exclusion of red blood cells) from mice exposed to filtered air or ozone (0.5, 1.0, 2.0, or 3.0 ppm) was performed to determine whether there was a relationship between ozone dose and percent cell death (Fig. 4). Values were normalized to control mice, and a significant increase in the percentage of cells that were permeable to trypan blue dye was observed in mice exposed to 3.0 ppm ozone.

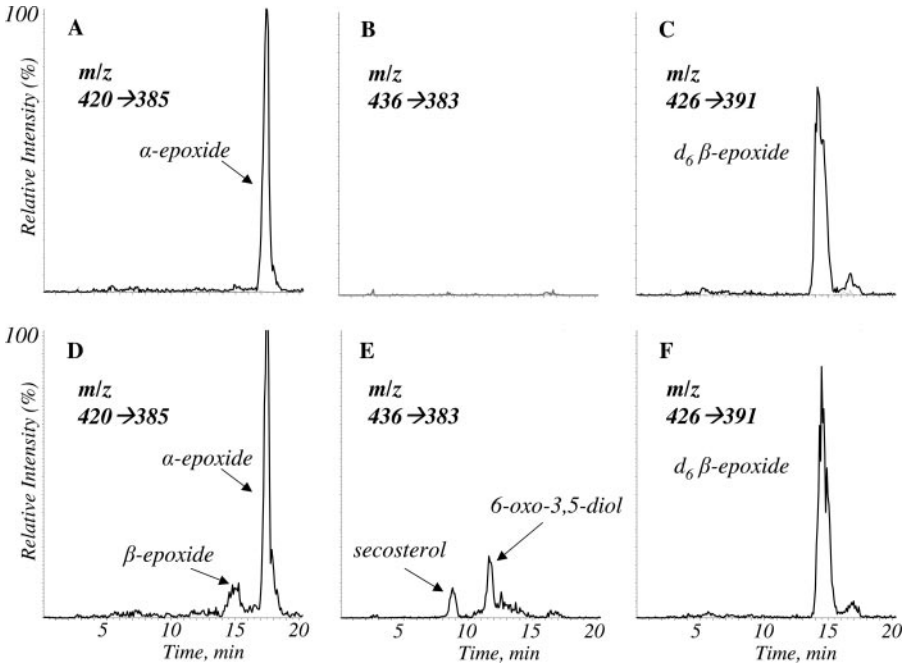


Fig. 1. Representative data for the increase of cholesterol ozonides in the BAL fluid from a mouse exposed to ozone (1.0 ppm) as compared with a control mouse. A to C, representative MRM chromatograms of cholesterol-derived ozonides from the BAL fluid of the control mouse. A, MRM transition for the 5,6-cholesterol epoxides (420 → 385) yielded a single peak, with a retention time consistent with α-epoxide. B, MRM transition for *seco*sterol and 6-oxo-3,5-diol yielded no peaks in the BAL from the control mouse. D to F, representative MRM chromatograms of cholesterol-derived ozonides from the BAL fluid of an ozone-treated mouse. D, MRM transition for the 5,6-cholesterol epoxides revealed two peaks, with the earlier peak corresponding to β-epoxide, as compared with the retention time of the deuterated standard (C and F). E, MRM transition for *seco*sterol and 6-oxo-3,5-diol (436 → 383) revealed that both compounds were formed in the BAL of the ozone-exposed mouse and that these products eluted with distinct chromatographic retention times.

Mass Spectral Analysis of Unknown in Vivo Oxysterol. Previous investigators have used *seco*sterol and norcholestane-6-carboxal as biomarkers of ozone exposure, since the formation of these products has been reported to be unique to the properties of ozone chemistry (Pryor et al., 1992; Wentworth et al., 2003). These two compounds have very different structures because one has an open B ring and the other has a closed ring system (Scheme 1). They are isobaric with a mass of 418 amu; however, the difference in lipophilicity due to ring structure leads to good separation on RP-HPLC. The *seco*sterol eluted from a C18 column at approximately 8 min with the gradient used in this study, whereas norcholestane-6-carboxal eluted at approximately 10.5 min. The identification of 6-oxo-3,5-diol as a major cellular metabolite of the cholesterol ozonide, β-epoxide, further complicated this situation because it was also isobaric with *seco*sterol and norcholestane-6-carboxal (molecular mass, 418 amu). The structure of 6-oxo-3,5-diol is very similar to norcholestane-6-carboxal, containing a fused ring system with hydroxyls at the carbon-3 and carbon-5 positions of cholesterol and a carbonyl at the carbon-6 position. As a result, norcholestane-6-carboxal and 6-oxo-3,5-diol eluted closely together on RP-HPLC, both with retention times near 10.5 min.

GC and electron ionization mass spectrometry were utilized to unambiguously determine whether the compound observed in vivo was norcholestane-6-carboxal or 6-oxo-3,5-diol. Standard preparations of norcholestane-6-carboxal and 6-oxo-3,5-diol were synthesized as described under *Materials and Methods* and then derivatized with BSTFA and subjected to GC/MS analysis. Both compounds yielded $M^{+\cdot}$ ions observed at *m/z* 562, which had distinct gas chromatographic elution times (Fig. 5). The retention time of 6-oxo-3,5-diol was 13.23 min. Extraction of *m/z* 562 from the norcholestane-6-carboxal spectrum yielded two abundant peaks at 11.96 and 12.84 min, which were also the most abundant peaks in the total ion chromatogram. The formation of two peaks was likely due to the formation of isomeric norcholestane-6-carboxal products, with stereoisomerism at carbon 6. A full electron ionization (70 eV) mass spectrum, from *m/z* 50 to 700, was collected for both compounds (Fig. 6).

Although the spectra of the two major peaks from norcholestane-6-carboxal were found to be similar (Fig. 6, B and C), there were large differences between the mass spectral data for norcholestane-6-carboxal and 6-oxo-3,5-diol. Several ions were consistent for all the spectra, including *m/z* 472, an $[M\text{-}90]^+$ ion that was characteristic for the loss of OTMS from the molecular ion (*m/z* 562). Ions at *m/z* 547, $[M\text{-}15]^+$, and *m/z* 457, $[M\text{-}105]^+$, presumably arose from the loss of a methyl group from an OTMS moiety of the molecular ion and the *m/z* 472 ion, respectively. A striking difference between the two spectra was the ion at *m/z* 382, $[M\text{-}180]^+$, which was consistent with the loss of both OTMS groups from the molecular ion. This was a prominent ion in the spectra of norcholestane-6-carboxal but not in the spectrum of 6-oxo-3,5-diol, suggesting that loss of the second OTMS group from this compound was hindered. This was consistent with the proposed structures of the ether trimethylsilyl derivatives of 6-oxo-3,5-diol and norcholestane-6-carboxal (Fig. 5), since there are fewer protons adjacent to the OTMS group at the carbon-6 position of 6-oxo-3,5-diol. The most remarkable contrast between the two spectra was the presence of an ion at *m/z* 321, which was the most abundant ion in the mass spectrum of norcholestane-6-carboxal. This ion was not observed in the mass spectrum of 6-oxo-3,5-diol.

The lung homogenates from mice exposed to 3.0 ppm ozone were pooled and injected onto RP-HPLC. The fractions containing the compound with a molecular mass of 418 amu ($[M+NH_4]^+$ ion, *m/z* 436), which eluted between 10 and 12 min, were collected for derivatization with BSTFA for GC/MS analysis. The electron ionization (70 eV) GC/MS spectrum (Fig. 6D) was consistent with the spectrum of 6-oxo-3,5-diol. Comparative chromatograms for the molecular ion, *m/z* 562, further supported the identification of the in vivo compound as 6-oxo-3,5-diol (Fig. 5C). The in vivo unknown yielded a single peak, observed at *m/z* 562, with a retention time of

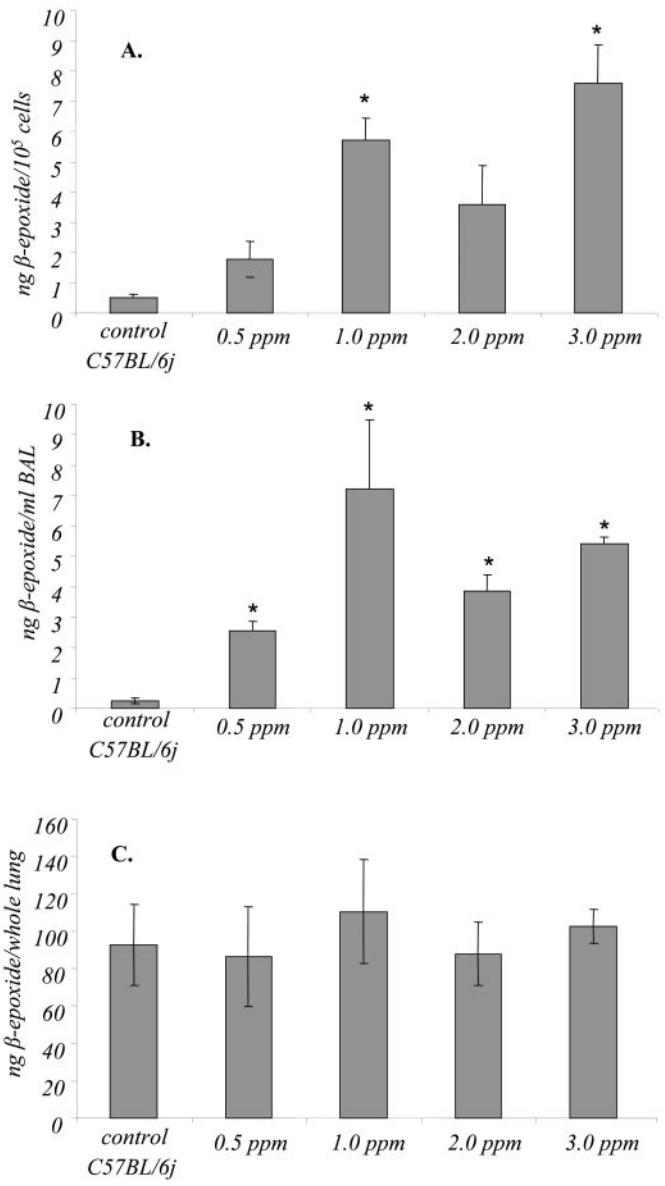


Fig. 2. Formation of β-epoxide in vivo following ozone exposure. Ozone-sensitive C57BL/6J mice were exposed to filtered air or to increasing concentrations of ozone (0.5, 1.0, 2.0, or 3.0 ppm). A, nanograms of β-epoxide found in bronchoalveolar lavaged cells, normalized for the number of cells recovered in the lavage. B, nanograms of β-epoxide detected in the lavage supernatant (nanograms per milliliter). C, nanograms of β-epoxide detected in whole-lung homogenate. For samples that were below the limits of detection in this quantitative assay, values of 0.16 ng per sample were assigned. $*$, $p < 0.05$ compared with control mice ($n = 4$).

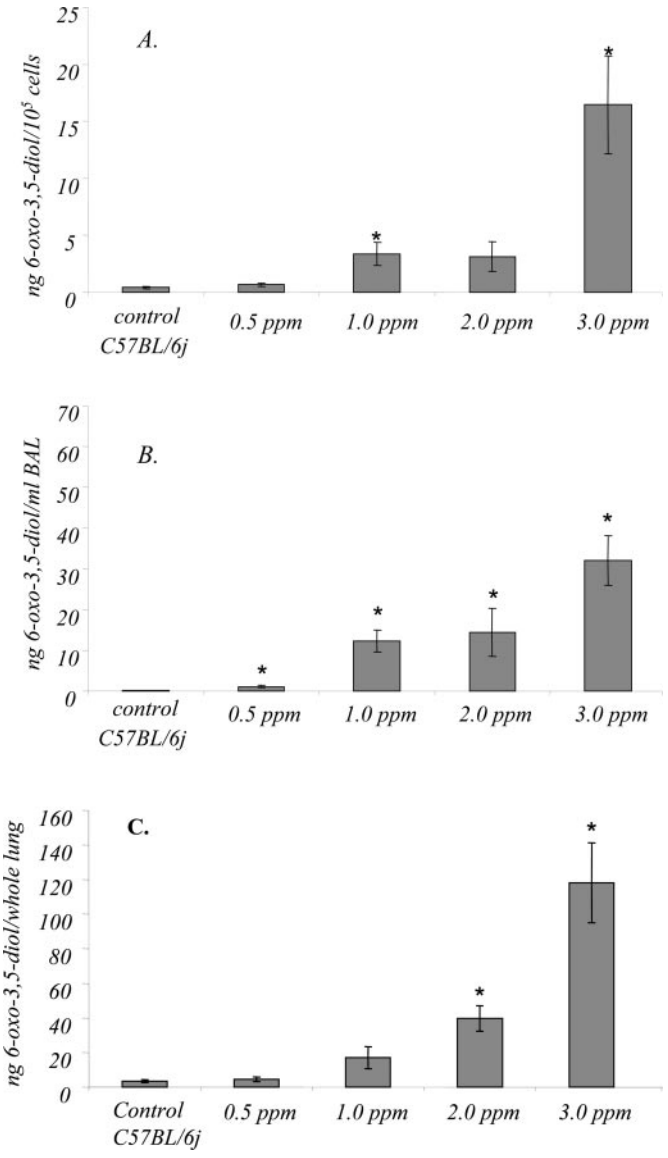


Fig. 3. Formation of 6-oxo-3,5-diol in vivo following ozone exposure. The ozone-sensitive mouse strain, C57BL/6J, was exposed filtered air or increasing concentrations of ozone (0.5, 1.0, 2.0, or 3.0 ppm). A, nanograms of 6-oxo-3,5-diol found in bronchoalveolar lavaged cells, normalized for the number of cells recovered in the lavage. B, nanograms of 6-oxo-3,5-diol detected in lavage supernatant (ng/ml). C, nanograms of 6-oxo-3,5-diol detected in whole-lung homogenate. $*$, $p < 0.05$ compared with control mice ($n = 4$).

13.22 min (compared with 13.23 min for synthetic 6-oxo-3,5-diol).

Discussion

Previous reports have suggested that cholesterol epoxide levels are higher in the lung than in any other tissue, but its formation was assumed to be a result of free radical-based mechanisms of peroxidation. The pollutant gas NO_2 has been found to induce peroxidation; therefore, not surprisingly, epoxide formation was observed after administration of NO_2 to rats (Sevanian et al., 1979). In those studies, rats were exposed to 6 ppm NO_2 for 48 h, which far exceeds the National Ambient Air Quality Standards of 0.053 ppm (U.S. Environmental Protection Agency, 2001). Even at this high NO_2 concentration, the levels of epoxide in BAL were only 2-fold higher than in filtered air-treated controls. In mice treated with 0.5 ppm ozone for 3 h (National Ambient Air Quality Standards of 0.12 ppm for 1 h) (U.S. Environmental Protection Agency, 2001), there was nearly a 20-fold increase in the level of epoxide found in both the fluid and cells of BAL. The unique chemical reactivity of ozone may be the reason for this difference. Ozone can initiate free radical production (Pryor et al., 1994), and the β-epoxide observed in vivo could be derived from lipid peroxidation mediated by this process. Additionally, ozone can act directly to form epoxides (Bailey et al., 1985), and as discussed previously (Pulfer and Mur-

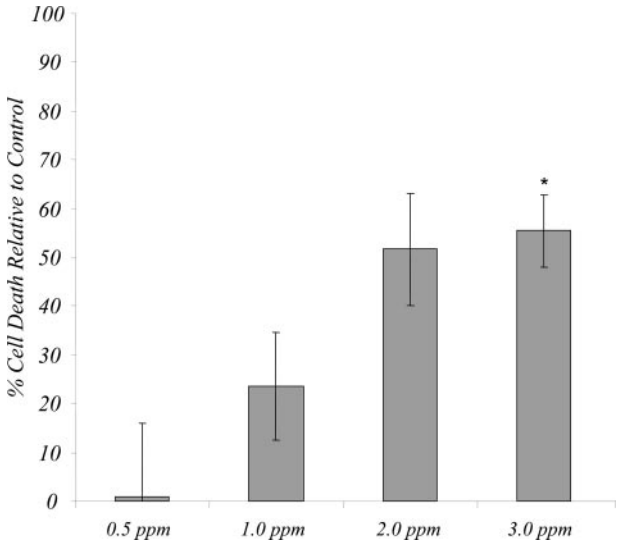


Fig. 4. Trypan blue exclusion analysis of cells recovered from bronchoalveolar lavage. Percent cell death for lavaged cells from mice exposed to 0.5, 1.0, 2.0, or 3.0 ppm ozone were normalized to those from control mice and expressed as percent cell death relative to control. *, $p < 0.05$ compared with control mice ($n = 4$).

phy, 2004), this mechanism of ozone chemistry becomes more prominent when ozone attacks compounds with a sterically hindered double bond, such as cholesterol.

The rationale for choice of the levels of ozone exposed to mice in these studies was based on previous work (Park et al., 2004a,b), which studied alterations in airway responsiveness, neutrophilic inflammation, changes in BAL protein control, and changes in epithelial cell integrity. These changes were shown to be very ozone dose-dependent because changes in airway function and epithelial cell damage were seen at 2.0 ppm ozone exposure for 3 h; at 1.0 ppm, these changes were much less obvious, although lung neutrophilic inflammation persisted. At 0.5 ppm ozone, there were little discernible effects in the lung as assessed by any of these parameters.

It is important to note that activated neutrophils recruited to the lung after ozone exposure could be responsible for secondary formation of β-epoxide and 6-oxo-3,5-diol. The elevated levels of these products for 24 h after ozone exposure suggest that continued formation due to neutrophil action may occur. Additional studies where neutrophils are depleted may shed some light on this question, since depletion does not affect changes in airway function or epithelial damage induced by ozone (Park et al., 2004a). Activated neutrophils can initiate free radical chemistry, which would potentially yield these products; however, recent studies have suggested that neutrophils may also generate ozone in vivo (Babior et al., 2003). To distinguish between ozone-generated products and free radical-mediated production of oxidized cholesterol products by neutrophils, unequivocal detection of 5-hydroperoxy-B-homo-6-oxa-cholestan-3β,7a-diol or *seco*sterol would be required because these are unique products from the reaction of ozone with cholesterol.

Auto-oxidation of cholesterol during work-up of samples has been shown to interfere with quantitative analysis of cholesterol epoxides (Wasilchuk et al., 1992). This may explain why a dose response was not seen in the lung homogenate samples. One suggested solution to this problem with sample preparation is to add deuterated cholesterol to samples prior to work-up to determine whether deuterated auto-oxidation products are formed. However, this was not compatible with the quantitative method in this study, which employed deuteratium-labeled internal standards. Therefore, the best way to quantitate epoxide formation may be to quantitatively assess the formation of 6-oxo-3,5-diol. Formation of this β-epoxide metabolite likely requires living cells, since it is thought to form via enzymatic mechanisms and is not formed in media. The mouse lung homogenates and BAL fluid were placed in organic solvents immediately, which inactivated enzymatic activity and prevented conversion of any β-epoxide formed during sample work-up to 6-oxo-3,5-diol. Consistent with this, the 6-oxo-3,5-diol seemed to better correlate with ozone exposure than the epoxide itself, as seen by the dose-response curve in lung homogenate (Fig. 3).

The levels of 6-oxo-3,5-diol in the lung homogenate from control mice were very low, ranging from 1.5 to 5 ng in whole lung, and there was a significant increase after ozone exposure, reaching levels of greater than 180 ng in some samples. An observed parallel change in β-epoxide levels would be expected since it is the precursor for 6-oxo-3,5-diol formation. Surprisingly, the levels of β-epoxide in the control mouse lungs were very high, ranging from 69 to 120 ng in whole lung, and there was not a significant increase in β-epoxide levels after ozone exposure. Therefore, there was an abundant amount of β-epoxide in control lungs that did not undergo conversion to 6-oxo-3,5-diol. The unambiguous structural determination of 6-oxo-3,5-diol in the in vivo samples excluded the possibility that another structurally similar cholesterol ozonolysis product was increasing in the ozone-treated mice lungs.

The absence of measurable metabolites in the lungs and BAL fluid of filtered air-treated mice suggested that either the β-epoxide measured in these samples was formed due to auto-oxidation during sample preparation or that there was a compartmentalization of the epoxycholesterol that prevented its metabolism via a cholesterol epoxide hydrolase. A distinctly active cholesterol epoxide hydrolase activity has been reported to exist (Watabe et al., 1986), which acts preferentially on 5,6-cholesterol epoxides; however, the protein(s) responsible for this activity have not been identified (Fretland and Omiecinski, 2000). As a result, it is not possible to determine which of the 40 different cell types in the lung could potentially metabolize β-epoxide. The activity of cholesterol epoxide hydrolase in lung microsomal fractions is lower than that of liver microsomal fractions (Sevanian et al., 1980), suggesting that only a fraction of the cells in the lung possess this activity. Another possibility was that induction of enzymes to metabolize the epoxide might occur during ozonolysis. Further study of the formation of β-epoxide metabolites and the enzymes involved in their formation is thus warranted.

The levels of 6-oxo-3,5-diol and β-epoxide in control cell extracts were below the limits of detection in this assay. These samples were therefore assigned quantitative values of <0.16 ng of oxysterol. Since these samples contained fewer numbers of cells in BAL than the ozone-exposed mice, this might overestimate the levels of β-epoxide and 6-oxo-3,5-diol in the control samples. As a result, the levels of these oxysterols in mice exposed to low doses of ozone were not significantly increased compared with control mice.

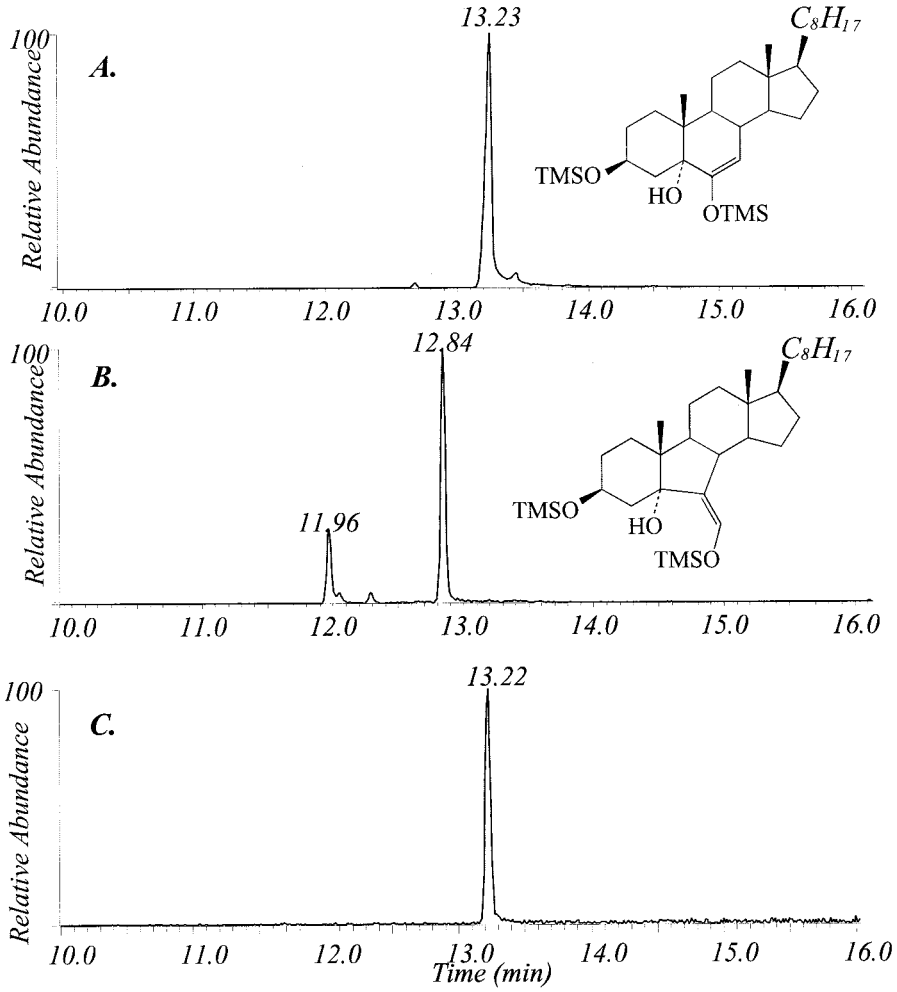


Fig. 5. Chromatograms from GC/MS analysis of: A, 6-oxo-3,5-diol; B, norcholestane-6-carboxal; and C, the in vivo unknown product with a molecular mass of 418 Da. The chromatograms represent extraction of *m/z* 562. The peaks observed corresponding to this ion were the most abundant peaks on the total ion chromatogram (data not shown).

It should be noted that the levels of β-epoxide in individual cells was likely quite variable because cells in different regions of the lung were presumably exposed to drastically different concentrations of ozone due to nonhomogenous distribution of this gas in the lung (Hu et al., 1994; Schelegle et al., 2001). On average, cells recovered from BAL of mice exposed to 0.5 ppm ozone contained 1.8 ng β-epoxide/10^5 cells. Considering that many of these individual cells may not have incorporated β-epoxide, there are likely some cells in this recovered lavage that have accumulated much higher concentrations of β-epoxide. When cultured bronchial epithelial cells were treated with 1 μM β-epoxide for 24 h, the amount incorporated into the membranes was only 12 ng/10^5 cells (data not shown). This concentration, which leads to potent suppression of cholesterol synthesis, is within the same range as the oxysterol levels in the membranes of bronchoalveolar lavaged cells from ozone-treated mice. Both β-epoxide and 6-oxo-3,5-diol have been shown to cause cell death in cultured lung epithelial cells (16-HBE) as assayed by trypan blue exclusion analysis (Pulfer and Murphy, 2004). The mechanism of action for β-epoxide and 6-oxo-3,5-diol is likely independent of cell type, and high concentrations of ozonized lipids could potentially lead to cytotoxicity in numerous cell types, including epithelial cells and macrophages. The overall increase in percentage of nonviable lavaged cells after ozone exposure paralleled the increased levels in the oxidized lipid products, consistent with a potential role of oxidized lipid products in ozone-induced cytotoxicity.

These oxysterols have not been studied extensively; therefore, they may bind to unknown receptors that initiate inflammatory signaling directly. For instance, 6-oxo-3,5-diol was reported to be a phorbol 12-myristate 13-acetate analog that binds to a cytosolic-nuclear receptor (Endo et al., 1993). Alternatively, β-epoxide and 6-oxo-3,5-diol could build up to concentrations lower than those that initiate cell death but still cause disruptions in the cellular membrane and alter signaling abilities. There are several studies that have provided evidence that cholesterol depleted cells have altered signaling properties due to disruption of lipid rafts (Pike and Miller, 1998; Westover et al., 2003). Decreased function in cells depleted of cholesterol is restored by cholesterol addition, but cholesterol analogs such as 4-cholesten-3-one do not restore activity (Nguyen and Taub, 2002). Increased proportional amounts of β-epoxide and 6-oxo-3,5-diol relative to cholesterol could lead to similar effects.

In summary, ozone exposure led to a dose-dependent formation of β-epoxide and 6-oxo-3,5-diol in the lungs of mice exposed to ozone concentrations from 0.5 to 3.0 ppm. These oxysterols have been observed to have cytotoxic effects in cultured lung cells, and they were formed in vivo at similar concentrations that could potentially lead to epithelial cell damage, which is an effect frequently associated with ozone exposure. Furthermore, this work revealed that molecular

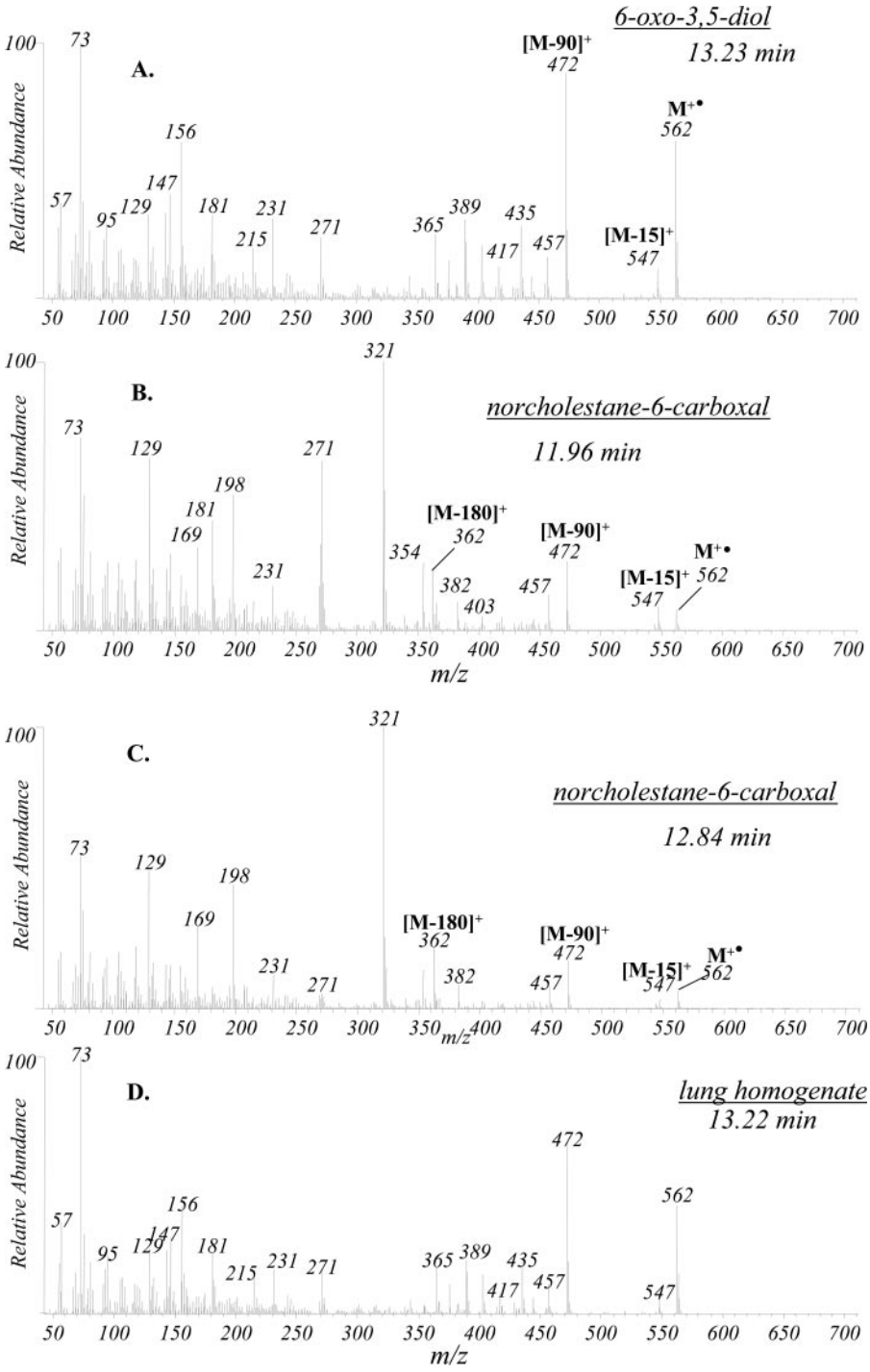


Fig. 6. Electron ionization (70 eV) mass spectrum of the trimethylsilyl derivatives of: A, 6-oxo-3,5-diol; B, norcholestane-6-carboxal (retention time 11.96 min); C, norcholestane-6-carboxal (retention time 12.84 min); and D, in vivo unknown.

weight analysis alone is not sufficient for identification of cholesterol ozonide products formed in vivo, since three products, 6-oxo-3,5-diol, *seco*sterol, and norcholestane-6-carboxal are isobaric with a molecular weight of 418 Da. This may be of particular relevance in studies suggesting that neutrophils generate ozone as revealed by formation of cholesterol ozonide products.

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