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Apoptosis induced by ozone and oxysterols in human alveolar epithelial cells

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Abstract

The mechanism of ozone-induced lung cell injury is poorly understood. One hypothesis is that ozone induces lipid peroxidation and that these peroxidized lipids produce oxidative stress and DNA damage. Oxysterols are lipid peroxide formed by the direct effect of ozone on pulmonary surfactant and cell membranes. We studied the effects of ozone and the oxysterol 5 β ,6 β -epoxycholesterol (β -epoxide) and its metabolite cholestan-6-oxo-3,5-diol (6-oxo-3,5-diol) on human alveolar epithelial type I-like cells (ATI-like cells) and type II cells (ATII cells). Ozone and oxysterols induced apoptosis and cytotoxicity in ATI-like cells. They also generated reactive oxygen species and DNA damage. Ozone and β -epoxide were strong inducers of nuclear factor erythroid 2-related factor 2 (Nrf2), heat shock protein 70 (Hsp70) and Fos-related antigen 1 (Fra1) protein expressions. Furthermore, we found higher sensitivity of ATI-like cells than ATII cells exposed to ozone or treated with β -epoxide or 6-oxo-3,5-diol. In general the response to the cholesterol epoxides was similar to the effect of ozone. The importance of understanding the response of human ATI-like cells and ATII cells to oxysterols may be useful for further studies, because these compounds may represent useful biomarkers in other diseases.

Keywords

alveolar type I-like cells; ozone; oxysterols; apoptosis

Introduction

Ozone is an extremely reactive gas and a major component of photochemical air pollution. Over 150 million people across the United States are exposed to ozone of unacceptable levels under the old 8 h standard of 0.08 ppm and more under the newer 0.075 ppm standard [1]. While ozone in the stratosphere plays an important role in preventing harmful ultraviolet radiation from reaching the surface of the earth, in the lower troposphere (ground level to 10 km), it is detrimental to health. The respiratory tract and cutaneous tissues are two major organs most directly exposed to oxidant pollutants [2]. Acute exposure to ozone causes damage to pulmonary epithelial cells. Furthermore, exposure to ambient levels of ozone can impair selective permeability of the epithelium [3]. Although much of the inhaled ozone reacts with

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the lining of the conducting airways, some can reach the proximal alveolar region when the exposure is at high concentrations (less than 0.5 ppm) or during exercise [4]. Ozone is also hypothesized to initiate intracellular oxidative stress through ozonide and hydroperoxide formation. These intracellular oxidants are likely to activate gene transcription through a redox-mediated signaling pathway that governs the cascade of injury, repair and other cellular responses associated with the oxidant burden [5]. However, the primary mechanism for observed ozone toxicity has not been fully defined.

Lung epithelial lining fluid, which contains pulmonary surfactant, is composed of almost 95% lipids; thus it has been proposed that ozone exerts its toxic effects via lipid mediators, which are formed during the interaction of ozone with lipids in the pulmonary surfactant [6]. Oxidized lipids can act as signaling molecules. Two targets for lipid peroxidation are cholesterol and phospholipids. Cholesterol, which is the most abundant neutral lipid in human pulmonary surfactant, has a double bond that is susceptible to attack by ozone. Several products have been reported to be formed during the reaction of ozone with cholesterol and the product yields have been shown to depend on ozonolysis conditions [6]. Cholesterol 5 β ,6 β -epoxide (®-epoxide) is a major product of cholesterol ozonolysis in a lipid environment and cholestan-6-oxo-3,5-diol (6-oxo-3,5-diol) is its major cellular metabolite [7]. These ozonized cholesterol products (oxysterols) are formed during exposure of pulmonary surfactant lung epithelial cells in mouse lung [6-8]. Pulfer and Murphy [6] showed that cholesterol epoxides are toxic to the lung epithelial cell line 16-HBE but direct studies on primary human epithelial cells have not been reported.

The DNA damage associated with ozone exposure could be the direct effect of ozone, but ozone is not thought to penetrate far into biologic fluids on cells [9]. However, these lipid peroxidative products such as oxysterols could generate reactive oxygen species (ROS) and produce DNA damage. Studies have shown that 8-oxoguanine is one of the most prevalent DNA adducts caused by ROS. Production of 8-oxoguanine leads to G-A transversion that is a common mutation in the *p53* gene [10].

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) has emerged as a critical regulator of the response to oxidative stress and is a key enhancer of a number of antioxidant and cytoprotective genes. The protein products of these genes are known as phase 2 enzymes, which directly destroy ROS, deactivate a large number of potentially harmful electrophilic molecules, and reduce oxidative stress [11]. Nrf2 regulates gene expression of heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1) and γ -glutamyl cysteine synthetase (γ -GCS), which are involved in the detoxification of ROS. HO-1 is induced by oxidative stress and its induction provides an important cellular defense mechanism against tissue injury. It was also reported that toxicity through oxidative stress, known to cause significant increases in cellular ROS concentrations, leads to the strong induction of HO-1 via the p38-Nrf2 signaling pathway [12,13]. However, Fos-related antigen 1 (Fra1) suppresses Nrf2-inducible *NQO1* and possibly γ -GCS expression. The fact that Fra1 cannot bind to DNA by itself suggests that interactions of Fra1 with other transcription factors, as well as their posttranslational modifications, may play a central role in the pathogenesis [14]. Heat-shock proteins (Hsps) are a group of molecular chaperone proteins that were shown to be induced by a variety of stresses. Although Hsps are cytoprotective, cells exposed to extreme or prolonged stresses undergo cell death by necrosis or apoptosis despite the expression of higher concentrations of Hsps. Therefore, expression of Hsp may be used as a sensitive biomarker when cells are placed under conditions of stress [15].

The purpose of our study was to determine if cholesterol epoxides induced cytotoxicity, apoptosis, and a cellular response similar to direct ozone exposure. We chose to use primary human alveolar epithelial cells and the targets that we have previously shown to be altered by

ozone exposure in rat alveolar epithelial cells [16]. Rat alveolar type I-like (ATI-like) cells are more susceptible to ozone than type II cells and are thought to be more sensitive to a variety of environmental toxins than alveolar type II (ATII) cells [16,17]. At relatively low concentrations, the centriacinar region (the junction between the alveoli and the conducting airways) is particularly affected by ozone, perhaps because, according to models, it receives a relatively large dose of ozone and because it has a large surface area covered by susceptible type I cells. After cessation of exposure and even during exposure, type I cells are replaced by proliferating ATII cells. Complete evolution of ATII cells to ATI cells does not occur during ozone exposure, regardless of the exposure period [18]. For these reasons, we have focused our study on the effect of ozone on human ATI-like cells. To our knowledge, no one has reported the effect of ozone and oxysterols in these cells. Moreover, we selected some relevant time points and assays to show that ATII cells obtained from the same lung donor are more resistant to exposure of ozone or treatment with oxysterols. However, human type I cells have not been isolated and cultured, so we chose to use ATI-like cells which are type II cells cultured to transdifferentiate into type I cells *in vitro* and express a similar but slightly different gene profile from isolated type I cells [19-21].

Materials and Methods

Isolation and culture of ATII and ATI-like cells

ATII cells were isolated from deidentified human lungs not suitable for transplantation and donated for medical research from the National Disease Research Interchange (Philadelphia, PA) and the International Institute for the Advancement of Medicine (Edison, NJ). The Committee for the Protection of Human Subjects at National Jewish Health approved this research. In the present study we selected donors with reasonable lung function with a PaO₂/FIO₂ ratio of > 250, a clinical history and x-ray that does not indicate infection, and limited time on the ventilator. We know the age, gender, race, smoking history, cause of death, very brief medical history, and medications at the time of death. Lung donors whose cells were used for experiments in this study were healthy non-smokers, Caucasians, 4 males and 3 females, with ages between 39-57 years.

The ATII cell isolation method has been published previously [22]. Briefly, the right middle lobe was perfused, lavaged, and then instilled with elastase (12.9 U/ml; Roche Diagnostics, Indianapolis, IN) for 50 min. at 37°C. The lung was minced and the cells were isolated by filtration and partially purified by centrifugation on a discontinuous density gradient made of Optiprep (Accurate Chemical Scientific Corp., Westbury, NY) with densities of 1.080 and 1.040 and by negative selection with CD14-coated magnetic beads (DynaL Biotech ASA, Oslo, Norway) and binding to IgG-coated petri dishes (Sigma Chemicals Inc., St. Louis, MO). The cells were counted and cytocentrifuge cell preparations were made to assess cell purity by staining for cytokeratin CAM 5.2 (Dako, Carpinteria, CA). The yield of ATII cells was ~300 × 10⁶ per isolation and the purity was ~80% before plating and over 95% after adherence in culture [19].

The isolated cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Franklin, MA), 2 mM glutamine (Thermo Scientific HyClone, Franklin, MA), 2.5 µg/ml amphotericin B (Mediatech Inc., Manassas, VA), 100 µg/ml streptomycin (Thermo Scientific HyClone, Franklin, MA), 100 U/ml penicillin (Thermo Scientific, Franklin, MA), and 10 µg/ml gentamicin (Sigma Chemicals Inc., St. Louis, MO). The cells were plated on millicell inserts (0.4 µm pore, 30 mm diameter, Millipore Corp., Bedford, MA) that had been previously coated with a mixture of 20% Engelbreth-Holm-Swarm (EHS) tumor matrix (BD Biosciences, San Jose, CA) and 80% rat-tail collagen in DMEM with 10% FBS and then cultured with 1% charcoal-stripped FBS (CS-FBS) along with 10 ng/ml keratinocyte growth factor (KGF; R&D Systems Inc., Minneapolis, MN), 0.1 mM

isobutylmethylxanthine (IBMX), 0.1 mM 8-Br-cAMP, and 10 nM dexamethasone (Dex; all from Sigma Chemicals Inc., St. Louis, MO) in addition to glutamine, amphotericin B, streptomycin, penicillin, and gentamicin as above [22].

To transdifferentiate type II cells into ATI-like cells, ATII cells were plated on rat tail collagen-coated tissue culture wells or glass coverslips in DMEM with 10% FBS and then cultured in DMEM with 5% FBS [19].

Ozone exposure

ATI-like and ATII cells were exposed *in vitro* to ozone in a computer-controlled humidified exposure chamber [23]. Ozone was generated by passing compressed medical grade oxygen through a cold-spark corona discharge ozone generator (Model OZ2SS-SS; Ozotech, Yreka, CA). Four specially designed 3.66 L glass chambers were used to expose cultured cells. One of these chambers was plumbed especially as a control chamber receiving only warm (37°C) and humidified air/CO₂ mixtures. The other three chambers were flushed with air/CO₂ mixtures containing ozone at concentrations 100 ppb, 200 ppb and 400 ppb. These concentrations in the sampled gas were measured by an ultraviolet ozone analyzer (model 400A, Advanced Pollution Instrumentation Inc., San Diego, CA) and controlled by a computerized system. For the 1 h exposure 300 µl serum free media was added to the cultured ATI-like cells on 6-well plates and the plates were rocked during the exposure. They were then cultured for 4 hours or 24 hours with regular serum-containing media. We also exposed ATI-like cells growing on a mixture of 80% rat-tail collagen and 20% EHS tumor matrix (Matrigel) to ozone (no fluid in the apical surface). However, we did not find any difference between results obtained with both systems (data not shown). Moreover, minimum fluid added to ATI-like cells and gentle rocking of well plates exposes one side of the culture well directly to ozone at a time. During the exposure of ATII cells growing on 30 mm diameter millicel inserts, the medium was removed from the apical compartment and 1 ml media was maintained in the basolateral part. Plates with cultured cells were gently rocked during the 1 h exposure. Cells were analyzed after 4 h and 24 h post one-hour exposure to 100 ppb, 200 ppb and 400 ppb ozone on the basis of our previous results [16,20,24].

Tested compounds

β-epoxide (Fig. 1A) was purchased from Sigma Chemicals Inc., St. Louis, MO (≥ 98%, CAS 4025-59-6) and 6-oxo-3,5-diol (Fig. 1B) was synthesized as previously described [6]. 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/H₂O/acetone (v/v/v; 4:1:0.5). The resulting cholestanetriol was extracted in dichloromethane and purified by reverse-phase high-pressure liquid chromatography (RP-HPLC). 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. β-epoxide and 6-oxo-3,5-diol were dissolved in ethanol for delivery to ATI-like and ATII cells and the final concentration of ethanol in the cultures did not exceed 0.04% (v/v). Cells were treated with 10 µM, 20 µM and 30 µM β-epoxide or 6-oxo-3,5-diol for 4 h and 24 h using media with serum [6]. ATI-like cells growing on 6-well plates were covered with 2 ml media containing oxysterols. We added 0.4 ml of medium containing oxysterols to the apical surface and 2 ml medium containing oxysterols to the basolateral compartment of ATII cell culture to maintain the same conditions for cell culture and cell treatments with these compounds.

We did not observe cellular detachment after 4 h post one-hour ATI-like cell exposure to ozone or after 4 h of treatment with oxysterols. We observed some floating cells after 24 hrs post one-

hour exposure to ozone or after 24 hrs of treatment with oxysterols. These cells were collected with attached cells for measurement of caspase 3 and caspase 7 activities and Western blotting.

MTT assay

The MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] assay was used to determine the concentrations of oxysterols used in this study. ATI-like cells were cultured on 96-well plates and incubated with β -epoxide or 6-oxo-3,5-diol for 4 h and 24 h. Briefly, 200 μ l of fresh DMEM and 50 μ l MTT reagent (2 mg/ml) were added to the cells and incubated for 1 h at 37°C. DMEM was then removed and 200 μ l DMSO was added followed by 25 μ l of Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 N NaOH). Samples were then read in triplicate at 570/630 nm using an automated microplate reader (SpectraMax 340PC; Molecular Devices Corp.). The percent viability was calculated by the formula: (sample absorbance)/(control absorbance) $\times$ 100%.

Methods to identify apoptosis and necrosis

a.) Propidium iodide and Hoechst 33342 double staining—To distinguish between live and necrotic cells we applied 10 mg/ml Hoechst 33342 and 1 mg/ml propidium iodide (both from Sigma Chemicals Inc., St. Louis, MO). In three independent experiments 300 cells were analyzed by fluorescence microscopy (Zeiss Axioskop 2, Carl Zeiss, Germany).

b.) Acridine orange and ethidium bromide double staining—Double staining using acridine orange and ethidium bromide (both from Sigma Chemicals Inc., St. Louis, MO) was performed as described previously [25]. Four cell stages were identified: (i) living cells (green nucleus with red-orange cytoplasm), (ii) early apoptosis stage (cell membrane still continuous but chromatin condensation with an irregular green nucleus is visible), (iii) late apoptosis (so called 'secondary necrosis' or 'apoptotic necrosis'- orange nuclei, fragmentation or condensation of chromatin is still observed), (iv) necrosis (uniform orange-colored cell nuclei). Following addition of 100 μ g/ml acridine orange and 100 μ g/ml ethidium bromide, 300 cells were immediately analyzed (taking into account these four stages) in three independent experiments by fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss, Germany).

c.) TUNEL assay—The TdT-mediated dUTP Nick-End Labeling (TUNEL; Promega, Madison, WI) assay was used to compare the ability of ozone, β -epoxide and 6-oxo-3,5-diol to induce apoptosis. This method was done as previously reported [26]. Briefly, ATI-like cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS and permeabilized with 0.2% triton X-100 (Sigma Chemicals Inc., St. Louis, MO). Then, slides with cells were incubated for 1 h at 37°C in a humid chamber in the presence of terminal deoxynucleotidyl transferase (TdT). In the positive control DNase (Promega, Madison, WI) was used, whereas no TdT was added in the negative control (data not shown). Cells were mounted with Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA). The analysis was carried out by fluorescence microscopy (Zeiss Axioskop 2, Germany). The percentage of apoptotic cells labeled with fluorescein (TUNEL-positive) was calculated per 10 high-power fields (magnification 10 $\times$ 40) [27].

d.) Caspase 3 and caspase 7 activity detection—Commercial Caspase-Glo 3/7 Assay kit (Promega, Madison, WI) was used to detect concentration- and time-dependent caspase 3 and caspase 7 activities in ATI-like cells exposed to ozone and treated with β -epoxide or 6-oxo-3,5-diol. The experiments were terminated after 4 h and 24 h. Samples were equilibrated to room temperature and for the apoptosis experiment 20,000 cells were used. Caspase-Glo 3/7 reagent was added for 1 h of incubation at room temperature. The luminescence signal was measured by a luminometer (Synergy HT, BioTek) according to the manufacturer's protocol.

Detection of 8-oxo-7,8-dihydro-2'-deoxyguanosine

ATI-like cells were fixed in 100% methanol and washed in PBS. After blocking with 3% normal donkey serum (Jackson ImmunoResearch; West Grove, PA) in PBS, the cells were incubated with goat antibody anti-OGG1/2 (L-19; Santa Cruz Biotechnology, Santa Cruz, CA) to detect 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) that is a sensitive marker of ROS-induced DNA damage. The secondary antibody, Alexa 488 anti-goat IgG (Invitrogen Corp., Eugene, OR) was applied with the cells for 1 h. Cells were mounted with Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA). The percentage of 8-oxo-dG-positive cells was calculated per 10 fields (magnification 10×40) [28] using fluorescence microscope (Zeiss Axioskop 2, Carl Zeiss, Germany).

Western blotting

Protein expression was measured by Western blotting according to protocols described previously [19]. Briefly, polyacrylamide gradient gels (8-16%; Invitrogen Corp., Carlsbad, CA) were run in tris glycine buffer to separate protein in the reduced state. Protein loading was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 40 kDa). Mouse anti-GAPDH was purchased from Abcam (Cambridge, MA), rabbit anti-HO-2 and mouse anti-HO-1 (Hsp32) were purchased from Assay Designs (Ann Arbor, MI). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse anti-Hsp70 (4E7), rabbit anti-Nrf2 (H-300) and rabbit anti-Fra1 (R-20). Horseradish peroxidase (HRP)-conjugated AffiniPure donkey anti-rabbit immunoglobulin (Ig) G and HRP-conjugated AffiniPure donkey anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA). The blots were then developed using an enhanced chemiluminescence (ECL) Western blotting kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Images obtained were quantitated using NIH Image 1.62 software.

Statistical analysis

Statistical differences among experimental groups were evaluated by one-way ANOVA using GraphPad Prism 4. A Dunnett's test was applied and a value of $p < 0.05$ was considered significant. Data are shown as the mean $\pm$ SEM from three independent experiments.

Results

Characterization of primary ATI-like and ATII cells

Isolated human ATII cells were cultured *in vitro* to achieve different differentiated phenotypes. The ATI-like and ATII cell cultures have been fully characterized previously [19]. To generate the ATI-like phenotype, the cells were cultured in 5% FBS. These cells are large flat cells that cover ~95% of the alveolar surface and through which gas exchange takes place. ATII cells displayed cuboidal shape, lamellar inclusions, microvilli, and tight junctions [19,22]. We used immunocytofluorescence for cytokeratin MFN116 and E-cadherin to further characterize their phenotype for these studies (Supplementary Fig. 1).

MTT assay

MTT assay is widely used to estimate cytotoxicity of tested compounds. ATI-like cells were treated with oxysterols for 4 h and 24 h. We selected 10 μ M, 20 μ M and 30 μ M concentrations of these compounds for our studies. The highest concentrations of β -epoxide or 6-oxo-3,5-diol induced 81.2% and 85.8% viability, respectively after 24 h (data in Supplementary Fig. 2).

Apoptosis and necrosis are induced by ozone and oxysterols

a.) Propidium iodide and Hoechst 33342 double staining—In order to distinguish between the necrotic and live cells, dual staining with propidium iodide and Hoechst 33342

dye was carried out (Supplementary Fig. 3). The percentage of necrotic ATI-like and ATII cells was calculated after 4 h and 24 h post one hour exposure to ozone or treatment for 4 h and 24 h with β -epoxide or 6-oxo-3,5-diol. We found a statistically significant difference after ATI-like cell exposure to 200 ppb and 400 ppb ozone. The highest percentage of necrotic cells was 10.8% for 400 ppb ozone after 24 h (Fig. 2A).

For ATI-like cells treated with β -epoxide we found a statistically significant increase in the number of necrotic cells after 4 h and 24 h treatment with 20 μ M and 30 μ M. The highest percentage of necrotic cells 11.2% was observed after 24 h treatment with 30 μ M β -epoxide (Fig. 2B). 6-oxo-3,5-diol induced a lower percentage of necrotic ATI-like cells than the β -epoxide (Fig. 2C). We found statistically significant differences only for 24 h treatment with 20 μ M and 30 μ M of this compound.

We also calculated the percentage of necrotic ATII cells after exposure to ozone or treatment with oxysterols. We did not observe a significant increase of necrotic ATII cells after 4 h and 24 h post one-hour exposure to ozone (Fig. 2D) and after treatment with β -epoxide (Fig. 2E) or 6-oxo-3,5-diol (Fig. 2F). Hence, type II cells are more resistant to the cytotoxic effects of ozone and the cholesterol epoxides than ATI-like cells.

b.) Acridine orange and ethidium bromide double staining—Acridine orange and ethidium bromide double staining allows distinguishing between viable, early and late apoptotic as well as necrotic cells (Supplementary Fig. 4). We calculated the percentage of cells in these stadia after ATI-like cell exposure to ozone and treatment with β -epoxide or 6-oxo-3,5-diol. The lowest cell viability 83.2% was found after 24 h post one hour ATI-like cell exposure to 400 ppb ozone. We also observed a higher percentage of apoptotic than necrotic cells increasing in a concentration- and time-dependent manner (Fig. 3A, B). After application of β -epoxide to ATI-like cell culture the highest decrease in viability 80.6% was found for 30 μ M after 24 h treatment (Fig. 3C, D). We also observed a higher percentage of necrotic than apoptotic cells after application 20 μ M and 30 μ M β -epoxide for 24 h.

The highest decrease in viability observed for 6-oxo-3,5-diol was 82.1% after 24 h treatment with 30 μ M of this compound (Fig. 3E, F). Our data indicate that ozone and oxysterols induced both apoptosis and necrosis and that these processes increased in a concentration- and time-dependent manner. Furthermore, ozone induced mainly apoptosis rather than necrosis contrary to β -epoxide and 6-oxo-3,5-diol.

c.) TUNEL assay—TUNEL assay also allows the detection of apoptotic cells (Supplementary Fig. 5). The highest percentage of apoptotic type I-like cells was observed after 24 h post one hour cell exposure to 400 ppb ozone (Fig. 4A). The lowest value was found after 4 h post one hour exposure of ATI-like cells to 100 ppb. A statistically significant difference was found after cell exposure to all applied concentrations of ozone after 24 h and for 200 ppb and 400 ppb after 4 h post one hour exposure.

The highest percentage of TUNEL-positive cells was found after 24 h treatment with 30 μ M β -epoxide and 30 μ M 6-oxo-3,5-diol. A statistically significant increase in apoptotic cells was observed after the application of 30 μ M β -epoxide for 4 h and all tested concentrations of this compound and 6-oxo-3,5-diol for 24 h (Fig. 4B, C). Our results indicate that ozone and both oxysterols induce apoptosis. Furthermore, the percentage of apoptotic cells was higher in cells exposed to ozone than treated with β -epoxide and 6-oxo-3,5-diol.

d.) Caspase 3 and caspase 7 activities—We determined caspase 3 and caspase 7 activities to estimate the apoptosis pathway induced by ozone, β -epoxide and 6-oxo-3,5-diol in ATI-like cells. Ozone did not induce statistically significant increase in their activities 4 h

or 24 h after exposure (Fig. 5A). β -epoxide induced an increase after 4 h treatment with all applied concentrations and after 24 h for 20 μ M of this compound (Fig. 5B). 6-oxo-3,5-diol induced statistically significant increases only at concentrations of 20 μ M and 30 μ M after 4 h treatment. We did not observe caspase 3 and caspase 7 activities after ATI-like cell treatment for 24 h with this compound (Fig. 5C).

Immunocytofluorescence analysis of 8-oxo-dG formation

We used analysis of 8-oxo-dG formation to detect ROS production after ATI-like cell exposure to ozone and treatment with oxysterols (Fig. 6). The percentage of 8-oxo-dG-positive ATI-like cells was statistically significant for ATI-like cells exposed to ozone or treated with oxysterols. These data are consistent with our other results that indicate that ozone and oxysterols are strong inducers of both ROS-induced protein expressions and apoptosis.

Nrf2 is increased in ATI-like cells in response to ozone, β -epoxide and 6-oxo-3,5-diol

The effects of ozone, β -epoxide and 6-oxo-3,5-diol were evaluated on the Nrf2 (~68 kDa), HO-1 (32 kDa), Fra1 (43 kDa) and Hsp70 (70 kDa) protein expressions in ATI-like cells. After ozone exposure Nrf2 expression was significantly increased at 24 h for 200 ppb and 400 ppb (Fig. 7A, B; Supplementary Fig. 6). Fra1 and Hsp70 expressions were increased at 24 h. HO-1 expression was found only after 4 h and 24 h post one hour ATI-like cell exposure to 800 ppb ozone (data not shown). We also estimated sensitivity of the ATII cells to ozone (Fig. 7C, Supplementary Fig. 7). Only Nrf2 expression was significantly increased after 24 h for 400 ppb and HO-1 expression was not detected (data not shown).

β -epoxide induced statistically significant expression of Nrf2, HO-1 and Fra1 in ATI-like cells (Fig. 8A, B; Supplementary Figs. 8, 9). After 4 h and 24 h treatment expression of Nrf2 increased even at 20 μ M. HO-1 expression was not detectable after 4 h of ATI-like cell treatment with this compound. However, we detected statistically significant late HO-1 expression for 20 μ M and 30 μ M β -epoxide. The highest concentration 30 μ M β -epoxide induced a statistically significant increase in Fra1 expression in ATI-like cells after 4 h and 24 h treatment. There was no statistically significant increase in Hsp70 expression. In ATII cells 30 μ M β -epoxide significantly increased expression of Nrf2 and Hsp70 after 24 h. We did not detect HO-1 expression in these cells (Fig. 8C, Supplementary Fig. 7).

After 4 h and 24 h of ATI-like treatment with 6-oxo-3,5-diol we observed lower expression of all analyzed proteins (Fig. 9A, B; Supplementary Figs. 8, 10) in comparison with β -epoxide. Only Nrf2 expression was significantly increased after 24 h treatment with 30 μ M of the former compound and the HO-1 expression was not detected (data not shown). In ATII cells 30 μ M 6-oxo-3,5-diol induced significant increases only Hsp70 expression (Fig. 9C, Supplementary Fig. 7).

The expression of HO-2 was constitutive in ATI-like cells and ATII cells and did not change as a consequence of ozone exposure or after treatment with β -epoxide or 6-oxo-3,5-diol (data not shown). Our results suggest time- and concentration-dependent ROS-sensitive protein expression after treatment with ozone and oxysterols. Moreover, we observed the lower sensitivity of ATII cells in comparison with ATI-like cells.

Discussion

Numerous publications report the effect of ozone and β -epoxide on different cell lines and very little is known about 6-oxo-3,5-diol. To our knowledge there are no prior studies of β -epoxide and 6-oxo-3,5-diol conducted in primary human alveolar ATI-like cells.

The ozone concentrations used here are probably significantly higher than exposure of human alveolar tissues to ozone. The ozone concentration decreases as it reacts with constituents along the conducting airways. The lung lining fluid layer is the most likely site of initial ozone interaction with absorption targets. Lung antioxidants, such as reduced glutathione, ascorbic acid, uric acid, and vitamin E, represent another line of defense against ozone injury and are present within the epithelial lining fluid [29]. The exact peak of ozone concentration during high ambient exposures during exercise is not known. The rationale for the choice of 100 ppb, 200 ppb and 400 ppb ozone in our studies was based on our previous results with rat alveolar epithelial cells *in vitro* [16,20,24]. Our results indicate that human normal primary alveolar cells are sensitive to low concentrations of ozone.

We found that ozone induced both apoptosis and necrosis in ATI-like cells as observed using Hoechst 33342 and propidium iodide (to detect necrosis) as well as acridine orange and ethidium bromide (to analyze necrosis and apoptosis) double stainings. Moreover, we also detected apoptotic cells in TUNEL assay. Our results are in agreement with Janic et al. [30] who reported that after the THP-1 macrophage cell line was exposed for 1 h to 100 ppb and 200 ppb ozone the cell viability was 95% and 68%, respectively as determined by trypan blue exclusion. Cheng et al. [10] observed 75% viability after 1 h A549 cell exposure to 120 ppb ozone. Studies indicate that exposure to 200 ppb ozone for 18 h caused A549 cell death both by apoptosis and necrosis [31]. Moreover, DNA damage induced by ozone in A549 and BEAS cells was also observed in comet assay [10,32].

In our study we found elevated caspase 3 and caspase 7 activities only in ATI-like cells exposed to oxysterols but not to ozone. These results suggest that ozone induces a non-caspase-mediated form of cell death in these cells. It was also reported that ozone did not induce caspase 3 activation in rat alveolar epithelial cells [16] and did not induce epithelial cell caspase-dependent apoptosis in rat terminal bronchioles [33].

We also detected ROS generation after ATI-like cell exposure to ozone by analysis of 8-oxo-dG formation, which is a biomarker of oxidative damage of DNA. Cheng et al. [10] reported that the 8-oxo-dG level increased in a concentration-dependent manner in A549 cells exposed to ozone and this increase was detected as low as 80 ppb. Moreover, ROS generation may be responsible for the increase of ROS-sensitive protein expression and serve as a mediator of apoptosis. This may explain the increased expression of oxidative stress-related Hsp70 and proapoptotic Fra1 in a concentration-dependent manner after 24 h post one hour exposure of AT-like cells to ozone. Wang et al. [16] also observed increases in Hsp70 and Fra1 in rat ATI-like cells exposed to ozone. To our knowledge there is only one report where the Nrf2 level was analyzed in relation to studying the effect of ozone and this protein was increased in mouse lungs after *in vivo* exposure [34]. In our study we found statistically significant Nrf2 expression after 24 h post one-hour ATI-like cell exposure to 200 ppb and 400 ppb ozone. However, we did not observe induction of HO-1 in these cells. This may be explained by the fact that the induction of oxidative stress by ozone was too low to detect this expression. First, we found increased expression of HO-1 after 4 h and 24 h post one-hour ATI-like cell exposure to 800 ppb ozone (data not shown). Second, *in vivo* results from mice exposed to ozone indicate that HO-1 is less responsive in the lung [2]. Third, ROS generation is an early event in cells, and then the intracellular ROS increase is detected by a Nrf2, which activates a set of antioxidant and anti-xenobiotic genes including HO-1. The induction of antioxidant genes represents late events in the antioxidant response [35]. Therefore in ATI-like cells exposed to ozone a strong induction of 8-oxo-dG may be an earlier stage than peak of HO-1 expression.

It was reported that β -epoxide induces apoptosis in human monocytic U937 cells. The effect of this compound was studied in comparison with a range of inhibitors of apoptosis. After a 24 h cell treatment with 30 μ M β -epoxide, the cell viability ranged from 62-80% in different

experiments using fluorescein diacetate and ethidium bromide staining [36]. These observations are in agreement with our results for the same time and concentration of this compound; 80.6% ATI-like cell viability was found using acridine orange and ethidium bromide double staining. However, for the same experimental conditions Ryan et al. [36] found the percentage of U937 apoptotic cells ranged from 11-16% and we observed only 6.5% and 7.6% apoptotic cells using acridine orange and ethidium bromide double staining and TUNEL assay, respectively. Moreover, in our study the number of necrotic cells 13.3% (acridine orange and ethidium bromide double staining) was higher than apoptotic cells. This may be explained by the higher sensitivity of ATI-like cells than U937 cells to β -epoxide because it was reported that high concentrations of the tested compound cause a switch from apoptosis to necrosis [37,38]. We also detected a caspase-dependent form of cell death in ATI-like cells treated with β -epoxide, which was also observed in U937 cell line [39]. Moreover, we found higher expression of proteins induced by the oxidative stress for β -epoxide than ozone. This stronger ATI-like cell response to this compound, which is an ozonation product of cholesterol, in comparison with their exposure to ozone can be explained by Pulfer and Murphy's [6] suggestion that direct exposure of cells to ozone led to accumulation of only nanomolar concentrations of β -epoxide in the cellular membranes. Nanograms of β -epoxide were also found in bronchoalveolar lavaged cells, lavage supernatant and whole-lung homogenate following *in vivo* ozone-sensitive mouse strain C57BL/6J exposure to ozone [7]. Our study found micromolar concentrations of oxysterols necessary for experiments. This is in agreement with Pulfer and Murphy [6] who used these levels for cytotoxicity and Kafoury et al. [6,40] who treated BEAS-2B cells with micromolar concentrations of an ozonized phosphatidylcholine lipid product and calculated the amount of lipid that partitioned into the cellular membrane. The micromolar concentration in media translated to low nanomolar concentrations in the cellular membrane supporting the use of these levels for *in vitro* studies [6].

A marked increase in ROS production and cytotoxicity in neuronal cells was found after treatment with β -epoxide and 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al [41]. It was reported that ROS have been implicated in oxysterol-induced apoptosis and their overproduction by 7-ketocholesterol represents the initiating event in the apoptotic process [42]. However, the mechanism of ROS generation induced by oxysterols within cells is not clear. In 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al treated neuronal cells, the increase in ROS production correlated with decrease in cellular glutathione and cell viability [43]. Another oxysterol, cholestane-3 β , 5 α , 6 β -triol causes oxidative damage, leading to the mitochondrial dysfunction, thus indicating a putative mechanism of apoptosis activation in isolated mice liver cells. It was suggested that this compound induced ROS generation probably by action on the antioxidant defense system because antioxidant butylated hydroxytoluene exhibited an inhibitory effect on mitochondrial oxidative damage [44]. Furthermore, Ryan et al. [45] found that the apoptotic process induced by 7 β -hydroxycholesterol is associated with a depletion of glutathione and an increase in superoxide dismutase activity, which are part of the enzymatic system that regulates the oxidative defense in cells. Oxysterols are capable of increasing the levels of the other antioxidant enzymes (e.g., glutathione peroxidase and catalase). These observations may explain increased Nrf2 and HO-1 expression found in our study after ATI-like cell treatment with β -epoxide. The *HO-1* gene expression is up regulated by stressors that increase the oxidant burden [46]. This correlation may be also confirmed by our results of immunocytofluorescence for 8-oxo-dG formation after ATI-like cells treatment with ozone and oxysterols.

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 phorbol 12-myristate 13-acetate has been shown to bind with high affinity. Furthermore, 6-oxo-3,5-diol has been used for photoaffinity labeling of this protein [6,47-49]. Similarly to β -epoxide, 6-oxo-3,5-diol was also formed in nanograms in

bronchoalveolar lavaged cells, lavage supernatant and whole-lung homogenate following *in vivo* ozone-sensitive mouse strain C57BL/6J exposure to ozone. However, the amount of this compound in nanograms was even lower than detected for β -epoxide [7].

In ATII cells ozone, β -epoxide and 6-oxo-3,5-diol were weaker inducers of protein expression and necrosis in comparison with ATI-like cells. Our data are in agreement with the results of Wang et al. [16] who demonstrated higher sensitivity of the rat ATI-like cells than the ATII cell phenotype in response to ozone. This may be caused by: (i) the physical shape of the cells and surface area; (ii) a higher mitochondrial in the ATII cells than ATI-like cells; (iii) ATII cells express components of the innate immune system which are considered cytoprotective; (iv) presence of KGF in ATII cell medium which activates survival pathways; (v) ATII cell express more antioxidant genes than ATI-like cells [16]; (vi) ozone and ozonation products of cholesterol β -epoxide and 6-oxo-3,5-diol may activate different signal pathways in these cells. While we have not studied the protein binding properties of either oxysterol used in these studies, there have been reports of β -epoxide binding to relevant proteins and including the scavenger receptor SR-AI/II and MARCO [50]. These molecules are hydrophobic and likely bind to various proteins.

In conclusion, the cytotoxicity observed in ATI-like cells provides evidence for the biological effects of ozone and oxysterols produced in ozonation reactions (Fig. 10). Although oxysterols have been extensively studied for their pivotal role in atherosclerosis, much less is known about their potential effects in the lung. The novelty of these studies lays in the demonstration that ozone, β -epoxide and 6-oxo-3,5-diol induces apoptosis and necrosis in human primary alveolar cells. For the first time we studied the effect of ozone, β -epoxide and 6-oxo-3,5-diol in human ATI-like cells. Moreover, we found that ATI-like cells are more sensitive than ATII cells. The importance of understanding the cellular response to oxysterols may be useful for further studies because these compounds and oxidative stress may represent useful biomarkers of numerous disease such as: neurological diseases, diabetes, familial combined hyperlipidemia, multiple sclerosis and cardiovascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

ATI-like cells	alveolar epithelial type I-like cells
ATII cells	alveolar epithelial type II cells
β-epoxide	5 β ,6 β -epoxycholesterol
6-oxo-3,5-diol	cholestan-6-oxo-3,5-diol
ROS	reactive oxygen species
Nrf2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H:quinone oxidoreductase 1
γ-GCS	γ -glutamyl cysteine synthetase
EHS	Engelbreth-Holm-Swarm
FBS	fetal bovine serum
CS-FBS	charcoal-stripped FBS
KGF	keratinocyte growth factor
IBMX	isobutylmethylxanthine
Dex	dexamethasone
RP-HPLC	reverse-phase high pressure liquid chromatography
8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
TUNEL	TdT-mediated dUTP Nick-End Labeling
TdT	terminal deoxynucleotidyl transferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

HRP

horseradish peroxidase

Fig. 1.
Chemical structures of the tested compounds. A - @-epoxide, B - 6-oxo-3,5-diol.

Fig. 2.

Cytotoxicity of ATI-like cells and ATII cells to ozone and the oxysterols. The percentage of necrotic ATI-like cells (A-C) and ATII cells (D-F) evaluated by Hoechst 33342 and propidium iodide double staining. A, D – necrotic cells 4 h and 24 h after exposure of cells to ozone; B, E – cells were treated with β -epoxide for 4 h and 24 h; C, F – cells were treated with 6-oxo-3,5-diol for 4 h and 24 h. Data represent results from three independent experiments ($p < 0.05$).

Fig. 3.

Evaluation of apoptosis and necrosis by acridine orange and ethidium bromide double staining in ATI-like cells. A - 4 h after post one hour ATI-like cells exposure to ozone; B - 24 h after post one hour cells exposure to ozone; C - ATI-like cells were treated with β-epoxide for 4 h; D - cells were treated with β-epoxide for 24 h; E - 6-oxo-3,5-diol was applied to ATI-like cells for 4 h; F - 6-oxo-3,5-diol was applied to cells for 24 h. Data represent results from three independent experiments.

Fig. 4.

Induction of apoptotic ATI-like cells in TUNEL assay. A - experiments were performed after 4 h and 24 h post one hour of ATI-like cells exposure to 100 ppb, 200 ppb and 400 ppb ozone; B – cells were treated with β -epoxide for 4 h and 24 h; C- ATI-like cells were treated with 6-oxo-3,5-diol for 4 h and 24 h. Data represent results from three independent experiments ($p < 0.05$).

Fig. 5.

Caspase 3 and caspase 7 activities in ATI-like cells. A – after 4 h and 24 h post one hour exposure to ozone; B – after 4 h and 24 h treatment with ®-epoxide; C – after 4 h and 24 h treatment with 6-oxo-3,5-diol. Data represent results from three independent experiments and are expressed in percentages of the control ($p < 0.05$).

Fig. 6. 8-oxo-dG formation in ATI-like cells. A - control cells, B - 24 h post one hour of cells exposure to 400 ppb ozone, C - cells treated with 30 μ M β -epoxide for 24 h, D - cells treated with 30 μ M 6-oxo-3,5-diol for 24 h; E – quantitation of 8-oxo-dG-positive cells after 24 h post one hour ATI-like cells exposed to 400 ppb and treatment for 24 h with 30 μ M β -epoxide or 6-oxo-3,5-diol. Data represent results from three independent experiments ($p < 0.05$).

Fig. 7.

Expression of stress related proteins after ozone exposure. Representative time and concentration course expression of proteins after 4 h (A) and 24 h (B) post one hour of ATI-like cells and after 24 h post one hour of ATII cells (C) exposure to ozone. Proteins were measured by immunoblotting. Lane 1, control; lane 2, 100 ppb; lane 3, 200 ppb; lane 4, 400 ppb.

Fig. 8.

Expression of stress related proteins after treatment with @-epoxide. Representative time and concentration course expression of proteins in ATI-like cells treated with @-epoxide for 4 h (A) and 24 h (B) and in ATII cells after treatment with this compound for 24 h (C). Proteins were measured by immunoblotting. Lane 1, control; lane 2, 10 μ M; lane 3, 20 μ M; lane 4, 30 μ M.

Fig. 9. Expression of stress related proteins after treatment with 6-oxo-3,5-diol. Representative time and concentration course expression of proteins in ATI-like cells treated with 6-oxo-3,5-diol for 4 h (A) and 24 h (B) and in ATII cells after treatment with this compound for 24 h (C). Proteins were measured by immunoblotting. Lane 1, control; lane 2, 10 μ M; lane 3, 20 μ M; lane 4, 30 μ M.

Fig. 10.

ATI-like cells response to ozone and oxysterols. Necrosis and apoptosis (chromatin condensation or fragmentation) were observed after cell exposure to ozone and treatment with α -epoxide or 6-oxo-3,5-diol. Moreover, the increased expression of Nrf2 and HO-1 proteins that are involved in the detoxification process were also detected.