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Original Contribution

Effects of ozone exposure on the ocular surface

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ABSTRACT

Changes in the ocular surface induced by ozone have received limited research attention. Here, we investigate the effects of ozone exposure on the integrity of the ocular surface, the production of inflammatory cytokines in tears, and changes in mucin-secreting cells in a mouse model. In addition, ozone-induced nuclear factor-κB (NF-κB)-mediated inflammatory processes were evaluated in cultured human conjunctival epithelial cells. In vivo, ozone induced the breakdown of corneal epithelial integrity, decreased the number of mucin-secreting cells, and induced the production of inflammatory cytokines, without altering tear volume. In vitro, ozone exposure led to increases in NF-κB nuclear translocation, κB-dependent transcriptional activity, NF-κB inhibitor α (IκBα) proteolysis, and expression of phosphorylated IκBα (p-IκBα), but did not cause cytotoxicity or cellular apoptosis. In addition, ozone induced the expression of inflammatory cytokines, Toll-like receptors, and C–C chemokine receptors, but decreased the expression of mucins. Furthermore, inhibition of NF-κB with pyrrolidine dithiocarbamate before exposure of cultured human conjunctival epithelial cells to ozone prevented changes in IκBα and p-IκBα levels in association with a decrease in the levels of inflammatory cytokines. Therefore, we conclude that ozone exposure interferes with ocular surface integrity and induces inflammation involving NF-κB-mediated processes at the level (and/or upstream) of IκBα. Understanding the role of ozone in the initiation of inflammatory processes on the animal ocular surface and in cultured human conjunctival epithelial cells can help elucidate the pathogenesis of ocular surface damage and suggest protective strategies for preserving a healthy ocular surface against ozone exposure.

Ozone (O_3), produced by reactions between nitrogen oxides and volatile organic compounds in a process catalyzed by ultraviolet light, is considered to be one of the most toxic air pollutants to which humans are routinely exposed [1]. The toxicity of tropospheric ozone, which can reach concentrations up to 0.8 ppm (ppm) in highly polluted metropolitan areas, is presumably due to ozone-mediated oxidative damage to various biomolecules [2,3]. Ozone can be rapidly converted into a number of reactive oxidant species and exerts its toxicity by reacting with cell proteins and lipids [4,5]. The respiratory tract, cutaneous tissue, and exposed ocular tissue are expected to be directly or indirectly affected by atmospheric ozone exposure.

Among wet-surfaced epithelia, corneal and conjunctival epithelia are the most exposed to external insults such as desiccation, pathogens, injury, and noxious stimuli. Thus, both secreted and membrane-tethered mucins (MUCs) of the corneal and conjunctival epithelia are necessary for protecting the ocular surface against various insults as well as conserving the major refractive surface of the eye. In the eye, gel-forming mucins are responsible for epithelium protection, maintenance of optical clarity, and refractive power [6]. As a surfactant for the ocular surface, they also allow an evenly spread tear film to wet the hydrophilic epithelium [6]. Goblet cells intercalated within the stratified conjunctival epithelia express and secrete gel-forming mucins, including MUC2 and MUC5AC [6]. MUC5AC, the most abundant gel-forming mucin, moves over the glycocalyx, collecting debris and pathogens and eliminating them [7,8]. The apical cells of the stratified epithelium of both cornea and conjunctiva express membrane-tethered mucins such as MUC1, -4, and -16 [6,9]. MUC4 acts as an antiadhesive molecule, lubricating the ocular surface and preventing infection by bacteria [10,11]. MUC16 plays a role in maintaining hydration and lubrication of the ocular surface, acting as a barrier against infectious agents and foreign particles [12]. These protective mechanisms also suggest a role for mucins in protecting the ocular surface against exposure to noxious stimuli such as ozone [6,13]. In addition to ocular mucins, both basal and reflex tearing might protect ocular surfaces against ozone exposure.

A number of studies have demonstrated effects of ozone exposure on the respiratory tract [14–21]. Ozone exposure has been reported to decrease pulmonary function, increase airway

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responsiveness, and induce airway inflammation in humans and experimental animals [22–28]. In addition, ozone exposure stimulates the activation of nuclear factor-κB (NF-κB) in human nasal epithelial cells and animal lungs [29–31]. Ozone exposure has also been shown to activate oxidative stress responses and inhibit wound healing in cutaneous tissues [32,33].

The transcription factor NF-κB has been demonstrated to play a pivotal role in modulating inflammatory responses and is required for maximal transcription of many inflammatory cytokines [34]. When activated by an external stimulus, NF-κB inhibitor (IκB) is phosphorylated by the IκB kinase complex and degraded by the proteasome. Freed NF-κB then quickly disassociates from its inhibitor and translocates to the nucleus, where it binds the κ enhancer in promoter regions of inducible genes, such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor-α (TNF-α), granulocyte–macrophage colony-stimulating factor, and intercellular adhesion molecule-1 (ICAM-1), thereby regulating their expression [35].

Toll-like receptor 4 (TLR4), which is rapidly upregulated on macrophages in response to pathogens, proinflammatory cytokines, and environmental stress, plays an important role in biological and functional responses of airway and lung tissue to ozone [36,37]. TLR2 also has been reported to contribute to functional responses of the airway to ozone [37].

Although ozone comes in direct contact with the ocular surface and usually causes ocular symptoms such as irritation, little is known about its effects on the ocular surface [38]. Based on the hypothesis that ozone induces an inflammatory response on the ocular surface, we evaluated corneal epithelial integrity, tear inflammatory cytokine levels, tear volume, and changes in mucin-secreting goblet cells to analyze the effects of ozone on the ocular surface of the mouse eye. We also investigated whether ozone exposure activates NF-κB-mediated inflammatory responses and induces the expression of mucins, inflammatory cytokines, TLRs, and C–C chemokine receptors (CCRs) in cultured human conjunctival epithelial cells. The effects of pharmacological intervention with the NF-κB pathway inhibitor pyrrolidine dithiocarbamate (PDTC) on inflammatory cytokine expression in response to ozone were also studied.

Material and methods

Animals

Thirty male ICR mice (Korea Laboratory Animal Co., Daejeon, Korea), 6–8 weeks of age, were randomly divided into three groups of 10 mice each. The mice were housed under a 12-h light/dark schedule (lights on at 6:00 AM, off at 6:00 PM) with ad libitum access to autoclaved food and water and were treated humanely and with regard for minimization of suffering. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System (10–105) and were conducted in accordance with the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology *Statement on the Use of Animals in Ophthalmic and Vision Research.*

Isolation and culture of conjunctival epithelial cells

During preparation of cornea buttons, human conjunctival tissues were saved and donated by the eye bank with patients' informed, written consent. Eight different conjunctivas were used in these studies. Donor demographics are summarized in Table 1. The entire conjunctiva was dissected approximately 2 mm lateral to the limbus of the cornea. Conjunctival cells were isolated by first incubating conjunctival specimens in phosphate-buffered saline (PBS; Millipore, Billerica, MA, USA) with 2.4 U dispase (Sigma–Aldrich, St. Louis, MO, USA) for 1 h in a 37 °C incubator. The loosened cells were then removed with a cell scraper, washed two times, seeded onto 60-mm plastic culture dishes, and incubated at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium used was keratinocyte cell basal medium (KGM-Gold; Clonetics Corp., Walkerville, MD, USA) supplemented with 5 mg/ml insulin (Clonetics), 0.5 mg/ml hydrocortisone (Clonetics), 0.5 mg/ml epinephrine (Clonetics), 10 ng/ml transferrin (Clonetics), gentamicin sulfate-1000 (Clonetics), 0.13 mg/ml bovine pituitary extract (Clonetics), and 10 ng/ml human epidermal growth factor (Sigma–Aldrich). The culture medium was changed 1 day after seeding and every other day thereafter until the cells reached 60–70% confluence (~5–6 days), at which time they were dissociated with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA; Clonetics). Each culture was split into three subcultures and seeded onto 10-cm plastic culture dishes. Passage-2 cells (10^5 cells/0.5 ml culture medium) were seeded onto 24-well plates (BD Falcon, Bradford, MA, USA).

Table 1
Demographics of donors.

Subject	Age (years)	Sex
1	40	M
2	43	F
3	35	F
4	46	M
5	37	M
6	32	F
7	47	F
8	44	F

M, male; F, female.

Ozone exposure

Mice were exposed to filtered room air (control), 0.5 ppm of ozone, or 2.0 ppm of ozone for 3 h in a whole-body exposure chamber. Exposure was performed routinely from 7:00 PM to 10:00 PM to ensure that the mice were in an awake–active state. Exposure was repeated every day for 2 weeks. Cultured conjunctival epithelial cells from each donor were exposed to 2.0 ppm of ozone for 0, 0.5, 1, 3, 5, or 8 h in an exposure chamber consisting of a Teflon-lined clear acrylic box (50 × 50 × 50 cm) with three holes: an ozone gas inlet, an ozone gas outlet, and a hole for monitoring ozone concentration. Each mouse was placed sideways on each hole so that its right flank was exposed to the inside of the chamber. Cells were placed in an environmental exposure system that allowed for simultaneous exposure of cells to ozone. We checked and adjusted the volume of the medium covering the surface of the culture plate. After ozone exposure, the cells were then cultured for 4 or 24 h with regular serum-containing medium. Ozone was generated with an OA-2 ozone generator (Ozone Engineering, Seoul, Korea). The concentration of ozone within the chamber was continuously monitored with a PortaSens II gas detector (Analytical Technology, Collegeville, PA, USA). Temperature (between 20 and 22 °C) and humidity (50 to 60%) were maintained at constant levels within the chamber.

Corneal fluorescein staining

Corneal fluorescein staining was measured by an observer blinded to the experimental groups before ozone exposure (baseline), after 1 and 2 weeks of ozone exposure, and 2 weeks after discontinuation of ozone exposure (4 weeks). Corneal epithelial integrity was determined by corneal fluorescein staining.

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