EFFECTS OF OZONE ON THE NASAL MUCOSA (EPITHELIAL CELLS)

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Abstract: Ozone is a significant public health concern worldwide. Despite increasing evidence for the role of the bronchial epithelial cells in the generation of proinflammatory cytokines there is little information on the biological relevance of ozone induced release of cytokines in nasal airway inflammation. We have investigated the effect of ozone on the nasal mucosa using immunohistochemical staining of nasal biopsies taken 6h after exposure to either 400 ppb ozone or filtered. We found that ozone significantly increases the number of neutrophils in the epithelium (p < 0.03), and expression of NF-kB (p < 0.03), TNF-a (p < 0.05), IL-1β (p < 0.03), IL-8 (p < 0.007), IL-6 (p < 0.02), GM-CSF (p < 0.02) and ICAM-1 (p < 0.01) in the epithelial cells 6h after exposure. Furthermore, we found a significant correlation between IL-8 expression and number of neutrophils (r = 0.85, p < 0.002) and NF-kB and TNF-a expression (r = 0.77, p < 0.009) in the epithelium.

These results suggest that ozone-induced inflammation of the nasal mucosa may be a consequence of increased synthesis and release of epithelial cell-derived cytokines and adhesion molecules which influence the activity of inflammatory cells.

Key words: ozone, cytokines, epithelial cells, NF-kappa B, nasal mucosa.

Introduction

Ozone is an air pollutant that has been recognized to be an important public health hazard. Epidemiological studies have demonstrated a clear association between increases in the ambient ozone concentration and impaired
lung function and increased bronchial hyperresponsiveness, both in asthmatic and nonasthmatic children [1]. Thus, ozone may play a significant role in the exacerbation of airway disease in asthmatics and could contribute to the overall increase in asthma morbidity [2].

Studies investigating the pathophysiological effects resulting from inhalation of ozone have demonstrated histological changes, including infiltration with inflammatory cells in the airways [3]. Studies in humans have demonstrated that exposure of healthy volunteers for 4–7 h to 80–200 ppb ozone induced epithelial damage with increased permeability, and increased number of neutrophils and several inflammatory mediators, in proximal airway lavage and bronchoalveolar lavage fluid collected 18–24 h after exposure [4]. Ozone has been shown to induce inflammatory changes in upper airways of both normal and allergic subjects. Graham and Koren [5] have demonstrated that ozone caused increased neutrophil content of nasal lavage fluid (NLF) from nonallergic subjects, indicating that ozone also induces nasal inflammation. Fischer and colleagues [6] have found increased amounts of eosinophil cationic protein and myeloperoxidase in NLF obtained from both allergic and normal children on days during which ambient ozone levels were increased.

Structural changes in the nasal mucosa have been demonstrated after ozone exposure of animals and humans. Prolonged exposure of primates to ozone at ambient levels results in marked changes in nasal epithelial morphology with squamous metaplasia in the anterior nasal cavity. One study of Mexican military recruits demonstrated significant alteration in nasal epithelial morphology among recruits assigned to high ozone areas compared with those assigned to low ozone areas.

Although the precise mechanism(s) by which ozone induces inflammation of the airways are not clear, there is increasing evidence that airway epithelium may play a vital role, since it can express and synthesize a large variety of proinflammatory mediators, including eicosanoids, cytokines and cell adhesion molecules, which influence the growth, differentiation, proliferation and activation of inflammatory cells [7].

In view of the immediate interaction of epithelial cells with ozone and their importance in modulating airway inflammation observed after inhalation of ozone, we postulate that this is result of increased synthesis and release of inflammatory cytokines from the airway epithelial cells.

Methods

Ten healthy, nonsmoking, nonatopic, volunteers (4 male, 6 female), 30.6 mean age (range 20–52) years were recruited. None had history of rhinitis, respiratory or other illnesses or were taking medication or antioxidants. They
had no respiratory tract infections for 6 weeks prior to or during the study period, had negative skin prick test to common airborne allergens. The study was approved by the local ethics committee and each subject gave their written informed consent.

**Study Design**

Each subject was exposed to filtered air and 400 ppb ozone for 2 hours at rest on two different occasions in randomized double-blinded controlled fashion, at least 2 weeks apart. Six hours after exposure either to filtered air or 400 ppb ozone nasal biopsies were taken.

**Ozone exposure**

Exposures were carried out using a purpose-built ozone exposure system in our laboratory. Ozone was produced using a generator (Triggered Ozone System, Glasgow, Model NVF 1/20 DE, manufactured by Ordup Maskin, Copenhagen, Denmark) capable of producing 5–30 mg/h of ozone.

**Nasal biopsy and tissue processing**

After prior local anesthesia with topical 4% lignocain containing 1:10,000 adrenaline, nasal biopsies were taken under direct vision from the inferior or inferomedial edge of the inferior turbinate, using Hartmann’s ear forceps (Medicon, Tuttinglen, Germany) as previously described [8].

The biopsies were immediately placed in ice-cooled acetone containing the protease inhibitors iodoacetamide (20 mM) and PMSF (2 mM) stored at -20°C for 24 h and then processed into water soluble GMA resin [9].

**Immunohistochemistry**

Processing of the biopsy in GMA and immunostaining were performed using a standard protocol as previously described. The following mouse IgG1 mAb were applied: AA1 to mast cell tryptase, UCHT1 to CD3 for pan T cells; anti-CD8 for the CD8+ T cell subset; anti-CD14 for macrophages; anti-Neutrophil Elastase for the neutrophils (all from Dako, High Wicombe, UK); anti-CD4 for CD4+ T cell subset (Becton Dickinson, Oxford, UK); EG2 to the cleaved form of ECP in activated eosinophils (Pharmacia, Milton Keynes, UK); anti-human GM-CSF and anti-IL-1b (Genzyme, West Malling, UK); 104-B11 to IL-6 (Dr. L.A. McNamee, Glaxo, Greenford, Middlesex, UK); anti-IL-8 (Biowhitaker House, Wokingham, UK); 12-H11 to 65p unit of NF-kB (Boehringer Meinheim, Lewes, UK); anti-RANTES and anti-TNF-a (R&D Systems Europe Ltd, Abingdon, UK), and RR1 to ICAM-1 (Dr. Rothlein, Boehringer Ingelheim, Ridgefield, CT).
Assessment and quantification of immunohistochemical staining

The length of overlying epithelium was calculated for each section using computer-assisted image analysis (Color Vision Software; Improvision, Birmingham, UK). Cells exhibiting positive immunoreactivity were counted and expressed as number of cells/mm length of basement membrane in the epithelium. The hue-saturation-intensity method of color image analysis was adopted for detection of the brown immunoperoxidase reaction product, as previously described, using the same image analyser. Repeated observations on single sections gave a standard error of 2% of the mean value. The color detection was programmed by first determining the hue-saturation-intensity values of the brown immunoperoxidase reaction product. The epithelial area was then outlined manually by use of a mouse facility in the software. The system then detected those pixels in the epithelial area that were within the appropriate range of hue-saturation-intensity values for the reaction product, and thus determined the percentage of the epithelial area that was stained. The whole of the nasal epithelium in each specimen was measured.

Statistical analyses

Statistical analyses were performed on SPSS (Windows version 6.1). Wilcoxon’s paired sign rank tests were used to compare the immunoreactivity of the nasal epithelium for the GM-CSF, IL-1β, IL-6, IL-8, RANTES, TNF-α, ICAM-1 and NF-kB and quantify the AA1, neutrophil elastase, CD3, CD4, CD8 and CD14 positive cells. Correlations were tested using Spearman’s rank correlation test. P values < 0.05 were considered significant.

Results

Nasal biopsies obtained 6 h after 400 ppb ozone exposure exhibited an increased number of neutrophils (neutrophil elastase positive cells) (Fig. 1) in the epithelium in comparison to biopsies obtained from same patients when they were exposed to filtered air. There were no epithelial changes in the number of mast cells (AA1+), activated eosinophils (EG2+ cells), total lymphocyte (CD3+ cells), and T cell subsets (CD4+ and CD8+ T-cells) and macrophages (CD14+ cells).

When using image analyzer there was significantly greater percentage of the epithelial area that was stained for NF-kB (p < 0.03), TNF-α (p < 0.05), IL-1β (p < 0.03), IL-8 (p < 0.007), IL-6 (p < 0.02), and GM-CSF (p < 0.02) and ICAM-1 (p < 0.01) in the biopsies obtained 6 h after exposure to 400 ppb ozone in comparison to filtered air exposure, (Table 1), but no difference in the expression of RANTES.
Figure 1 – Immunostaining of epithelium for Ne in patient exposed to 400 ppb ozone

Furthermore, staining of the epithelium for IL-8 showed a significant positive correlation \( r = 0.85, p < 0.002 \) with the number of neutrophils (Tab. 1). There was a significant correlation between staining of the epithelium for NF-kB and TNF-a \( r = 0.77, p < 0.009 \) (Tab. 1).

Table 1 – Таблица 1

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<th>Summary of the results</th>
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<td><strong>Epithelium (ozone v. air)</strong></td>
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<td>• NF-kB activated form ( p &lt; 0.03 )</td>
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<td>• TNF-a ( p &lt; 0.05 )</td>
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<td>• IL-8 ( p &lt; 0.007 )</td>
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<td>• IL-6 ( p &lt; 0.02 )</td>
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<td>• GM-CSF ( p &lt; 0.02 )</td>
<td>• GM-CSF ( p &lt; 0.02 )</td>
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<td>• Neutrophils ( p &lt; 0.03 )</td>
<td>• Неутрофили ( p &lt; 0.03 )</td>
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<th><strong>Correlation</strong></th>
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<td>• IL-8 (ozone) and Neutrophils (ozone) ( r = 0.85, p &lt; 0.002 )</td>
<td>• IL-8 (озон) и неутрофили (озон) ( r = 0.85, p &lt; 0.002 )</td>
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<td>• NF-kB (ozone) and TNF-a (ozone) ( r = 0.77, p &lt; 0.009 )</td>
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Discussion

This study has demonstrated that exposure of normal subjects for 2 h at rest to 400 ppb ozone leads to significant upregulation of NF-κB, IL-8, TNF-α, IL1-β, GM-CSF, IL-6 and ICAM-1 in the nasal epithelium. Additionally, we found significantly increased number of neutrophils in the epithelium of the patients when they were exposed to 400 ppb ozone. Furthermore, we found a significant correlation between IL-8 expression and number of neutrophils and positive correlation between the expression of NF-κB and TNF-α in the epithelium.

Although a large number of both controlled laboratory and epidemiologic studies have proved the adverse influence of ozone on the lower respiratory tract, little attention has been drawn to ozone influence on the upper respiratory tract. Laboratory studies have demonstrated that ozone is taken up to 40% by the nasal mucosa. This high nasal uptake and the fact that humans are predominantly nose breathers indicates that a possible adverse effect can be produced at this level. In fact, a few number of controlled studies have demonstrated this possibility [10].

Recent studies have shown that epithelial cells can produce diverse compounds that can contribute to inflammation and airway hyperreactivity, including: prostaglandins and leukotriene [11], platelet-activating factor [12], fibronectin [13], cytokines [14], hematopoietic growth factors, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, and macrophage colony-stimulating factor, and chemotactic factors for other cells. Thus, epithelial cells may directly contribute to the airway inflammation observed in ozone exposed humans and animals.

As potent oxidant, ozone is capable of reacting with a variety of extracellular and intracellular biomolecules, particularly those containing thiol or amine groups or unsaturated C = C bonds. Several enzymes of intermediary metabolism, and antiproteinase function [15] have been shown to be altered or inactivated by ozone in vivo and in vitro. Ozone reacts with glutathione, ascorbate, and uric acid, which are present in airways surface liquid and may serve a protective, ozone-scavenging function. The cytotoxicity of ozone is largely due to its interaction with unsaturated lipids which generate damaging free radicals or toxic intermediate products such as hydrogen peroxide (H₂O₂) and aldehydes [16]. Oxygen free radicals and their by-products that are capable of causing oxidative damage, collectively referred to as reactive oxygen species (ROS), may be cytotoxic when produced in huge excess whereas produced in moderate concentration can influence gene expression as well as posttranslational modification of proteins. One eukaryotic inducible transcription factor activated by ozone or its intermediate product H₂O₂ is NF-κB. The major site of interaction between the nasal mucosa and inhaled oxidants (ozone) is the nasal
epithelium, therefore we believe that ozone and H$_2$O$_2$ are able to produce active NF-kB. We found significantly higher expression of activated form of NF-kB in the epithelium of the patients exposed to 400 ppb ozone in comparison to filtered air exposure. Haddad, et al. demonstrated increased DNA-binding activity of NF-kB after ozone exposure in rat lung tissue. There are also reports that ozone leads to NF-kB activation. Ozone itself or via its intermediate product H$_2$O$_2$ can trigger reaction that leads to the degradation of the bound IkB protein. Loss of the inhibitory IkB protein bound to the protein complex p50-p65 is followed by rapid translocation of the p50-p65 heterodimer to the nucleus. The transcription is switched on after the binding of p50-p65 to cis-acting kB sites in the promoters and enhancers gene. NF-kB is implicated in the activation of some gene families encoding proinflammatory cytokines (IL-1β, TNF-α, IL-6, IL-2, GM-CSF, MC-SF, GC-SF), chemokines (IL-8, MIP-1α, MCP-1, Gro-α, -β and -g and eotaxin), inflammatory enzymes (iNOS, inducible cyclooxygenase-2, 5-lipoxygenase, cytosolic phospholipase A$_2$), adhesion molecules (ICAM-1, VCAM-1, E-selectin) and receptors (IL-2R, T-cell receptor) [17]. We found upregulated expression of IL1-β, TNF-α, IL-6, IL-8 and GM-CSF in the nasal epithelium of the patients exposed to 400 ppb ozone. Increased expression of IL-8 led to increased number of neutrophils in the epithelium and submucosa and we found a significant correlation between IL-8 expression and number of neutrophils in the epithelium. IL-8 is a major cytokine in the recruitment of neutrophils to areas of inflammation, activation of neutrophil degradation and respiratory burst, thus contributing to the ozone induced inflammation. Neutrophils release their enzyme damaging the epithelial cells, thus contributing to the epithelial sloughing. Tarraf, et al. demonstrated increased release of IL-8 by both, cultured human bronchial epithelial monolayers and dispersed cell preparations after 6h exposure to different ozone concentrations [18].

Rusznak et al. [19] have demonstrated that exposure of human bronchial epithelial cells for 6 h to 10–500 ppb ozone leads to significant epithelial cell damage and to release of IL-8, GM-CSF, TNF-α and sICAM-1. Both in vivo and in vitro studies have demonstrated that human nasal epithelial cells can generate several cytokines including IL-1β, TNF-α, IL-6, IL-8, GM-CSF, and the C-C chemokine RANTES. We found that ozone upregulates the expression of IL-1β, TNF-α, IL-6, IL-8 and GM-CSF 6 h after the exposure most likely via activation of NF-kB. The activation of NF-kB leads to a coordinated stimulation of the expression of the genes for E-selectin, ICAM-1, IL-8, TNF-α and IL-1β in the endothelial cells resulting in recruitment and activation of neutrophils (our unpublished data). Products of the genes that are regulated by NF-kB also cause the activation of NF-kB. The proinflammatory cytokines IL-1β and TNF-α both activate and are activated by NF-kB. This positive regulatory loop may amplify and perpetuate local inflammatory responses. We found a significant positive correlation between expression of TNF-α and NF-kB in the epithelium 6 h after exposure to 400 ppb ozone.
These results suggest that in the airways the initial response to oxidative stress may be to induce NF-kB responsive genes. Because of their instability and their ability to damage cells and tissues, the control and removal of free radical species is important [20]. Nitric oxide is able to interact with ROS and thus reduce their activity. As such the induction of iNOS gene expression in the airway epithelium, which form the most important barrier to oxidants in the respiratory tract, may play an important role in the regulation of oxidative stress.

We also found significant upregulation of ICAM-1 expression in the epithelium 6 h after ozone exposure probably via activation of NF-kB. It is well documented that activation of NF-kB leads to an increase in the expression of the genes for adhesion molecules i.e. ICAM-1. Thus, ICAM-1 can contribute to the increased binding of neutrophils via its ligands CD11b to the nasal epithelial cells permitting them to release its mediators damaging the cells and perpetuating the local inflammatory response. There are only two recent studies reporting that in vitro ozone exposed human tracheal epithelial cells and BEAS cells bind substantially more neutrophils than do air-exposed cells and that these may be regulated by adhesion molecules. Beck et al demonstrated that nasal epithelial cells when placed into culture respond to high level ozone (500 ppb) by generating ICAM-1, the major adhesion molecule for neutrophils and eosinophils in the airways. Taffaf et al have demonstrated increased expression of ICAM-1 by both, cultured human bronchial epithelial monolayers and dispersed cell preparations after 6h exposure to different ozone concentrations.

Acute exposure to ozone provokes an upper airway inflammatory response that includes infiltration of the nasal mucosa with neutrophils and mast-cells, release of proinflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8 and GM-CSF), expression of adhesion molecules ICAM-1 and maybe others all released by epithelial cells after activation of NF-kB mediated by ROS (Fig. 2).
Effects of ozone on the nasal mucosa (epithelial cells)


REFERENCES


Озонот претставува значаен здравствен проблем на секаде во светот. И покрај сч# поголемиот број докази за учеството на озонот во генерирањето на проинфламаторни цитокини во бронхијалните епителни клетки, постојат скуди податоци за неговата улога во индуцирањето на назалната инфламација.

Го испитавме ефектот на озонот врз назалната мукозa употребувајки техника на имнохистохемиско боение на назалните биопси земени по 6 часовна експозиција на пациентите на/или 400 ppb (делови од милијардата) озон или филтриран воздух. Констатиравме дека озонот сизинфикантно го зголемува бројот на неутрофилите во назалниот епител (p < 0,03) и експресијата на NF-κB (p < 0,03), TNF-α (p < 0,05), IL-1β (p < 0,03), IL-8 (p < 0,007), IL-6 (p < 0,02), GM-CSF (p < 0,02), и ICAM-1 (p < 0,01), во епителните клетки по 6 часовна експозиција на 400 ppb озон. Исто така констатиравме сизинфикантна корелација помеѓу експресијата на IL-8 и бројот на неутрофилите (r = 0,85, p < 0,002) и помеѓу експресијата на NF-κB и TNF-α (r = 0,77, p < 0,009) во назалниот епител.

Овие резултати сугерираат дека инфламацијата на назалната мукоза индуцирана од озонот можеби е последица на зголемената синтеза и ослободување на цитокини и атхезивни молекули од епителните клетки, кои влијаат на развојот на инфламаторната клеточна реакција.

Ключни зборови: озон, цитокини, епителни клетки, NF–κB, назална мукоза

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