

EFFECTS OF OZONE ON NASAL MUCOSA (ENDOTHELIAL CELLS)

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Abstract: After exposure to ozone, humans develop neutrophil infiltration of the nasal mucosa. To investigate the events contributing to inflammatory cell recruitment in the nasal mucosa we exposed 10 healthy nonsmoking volunteers to 400 ppb ozone or filtered air for 2h at rest on two separate occasions. Nasal biopsies were performed 6h after ozone/filtered air exposure. The nasal biopsies were embedded in glycol mathacrylate and immunostained for inflammatory cells, including neutrophils, mast cells, total T-cells (CD3), T-cell subsets CD8 and CD4, macrophages, eosinophils adhesion molecules (P-selectin, E-selectin, ICAM-1, VCAM-1), cytokines (TNF- α , IL-1 β , GM-CSF, IL-6), chemokines (IL-8 and RANTES), and nuclear factor NF- κ B. No significant changes were seen in the number of T-cells, and T-cell subsets, eosinophils, macrophages, or percentages of vessels expressing P-selectin, VCAM-1, GM-CSF, IL-6 and RANTES in the biopsies. The number of neutrophils and mast cells in the submucosa was significantly higher after ozone exposure ($p = 0.009$ and $p = 0.005$ respectively). The percentage of vessels expressing E-selectin ($p = 0.01$), ICAM-1 ($p = 0.005$), IL-8 ($p = 0.02$), TNF- α ($p = 0.02$), IL-1 β ($p = 0.009$), and NF- κ B ($p = 0.05$) increased significantly after ozone exposure as compared to filtered air exposure.

Exposure of normal subjects to ozone increases the expression of proinflammatory cytokines resulting in upregulation of IL-8 and adhesion molecules via activation of NF- κ B, leading to neutrophil infiltration in the nasal mucosa.

Key words: ozone, neutrophils, ICAM-1, cytokines, nasal mucosa.

Introduction

Ozone is a major component of photochemical airpollution produced from the interaction of sunlight, oxides of nitrogen, and volatile organic compounds. It is often associated with increased respiratory symptoms in allergic individuals during episodes of poor air quality. After exposure to ozone, humans develop increased levels of neutrophils in nasal lavage fluid [1–3] and increases in soluble components, such as albumin (a permeability marker) and histamine and tryptase (mast-cell degranulation markers) [3, 4]. Graham and Koren [2] reported a qualitative correlation between neutrophil increases in the lower airways as assessed by BAL and in the upper respiratory tract as assessed by nasal lavage. In a recent European field study, nasal lavage was performed on children at various intervals before, during, and after the season of highest ozone levels; a positive correlation was found between nasal lavage neutrophils and measured ambient ozone concentration [5]. It is likely that airway neutrophilia occurs as a result of recruitment from the microvasculature involving activation of leukocyte-endothelial adhesion molecules. Selectins play a pivotal role in the initial phase of adhesion [6]. This is followed by increased expression of members of the immunoglobulin super family, intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1) with up-regulation of the corresponding ligands, leading to firm adhesion of the leukocytes to endothelium and trans-endothelial migration. Exposure to ambient levels of ozone results in an up-regulation of ICAM-1 [7] in the vascular endothelium of the proximal airways mucosa associated with an increased expression of CD11b, a ligand for ICAM-1 on BAL neutrophils.

In order to elicit the events involved in ozone-induced inflammatory reaction, we obtained nasal biopsies 6h after controlled exposure to 400 ppb ozone for 2 h at rest.

Objectives of the study

We wanted to examine the effects of controlled exposure to 400 ppb ozone for 2 h at rest on healthy volunteers, especially on the production of pro-inflammatory mediators of the endothelial cell to be able to better understand the mechanism of ozone-induced mucosal inflammation.

Methods

Ten healthy, nonsmoking, non-atopic, volunteers (4 male, 6 female), mean age 30.6 (range 20–52) years were recruited. None had a history of rhinitis, respiratory or other illnesses or were taking medication or antioxidants. They had had no respiratory tract infections for 6 weeks prior to or during the study period, had negative skin prick tests 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 anaesthesia 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, Tuttlingen, 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 [8, 10]. The following mouse IgG1 mAb were applied: AA1 to mast cell tryptase (11), 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 Wycombe, 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-1 β (Genzyme, West Malling, UK), 104-

B11 to IL-6 (Dr.L.A. McNamee, Glaxo, Greenford, Middlesex, UK); anti-IL-8 (Biowhittaker House, Wokingham, UK); 12-H11 to 65p unit of NF- κ B (Boehringer Mannheim, Lewes, UK); anti-RANTES and anti-TNF- α (R&D Systems Europe Ltd, Abingdon, UK), and RR1 to ICAM-1 (Dr.Rothlein, Boehringer Ingelheim, Ridgefield, CT).

Assessment and quantification of immunohistochemical staining

Immunostaining cells were counted in the submucosa and areas, including smooth muscle, glands, large blood vessels, and torn or folded tissue within the section were excluded. The area of the submucosa where the cell count was performed were determined using computer-assisted image analysis (Color Vision Software; Improvision, Birmingham, UK), and the results were expressed as cells/mm² of the submucosa. Cytokines, chemokines, NF- κ B, and adhesion molecules in the submucosal blood vessels were quantified by expressing the proportion of vessels staining with mAB as percentage of the total number of vessels revealed by staining with pan endothelial mAB EN4 in adjacent sections (2 μ m apart) [10].

Statistical analysis

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-1 β , IL-6, IL-8, RANTES, TNF- α , ICAM-1 and NF- κ B 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, $p = 0.005$), and mast cells (AA1⁺, $p = 0.009$) in the submucosa in comparison to biopsies obtained from the same patients when they were exposed to filtered air. There was no significant difference in the numbers of total T-cells (including subsets), CD14⁺ cells, and activated eosinophils (EG2⁺ cells) in the submucosa (Tab 1a).
Table Ia

*Immunohistochemistry data (inflammatory cells)**

mAb	Air	Ozone	p-Value†
CD3	431.8 (145.5–990.3)	546.5 (148.4–1008.8)	NS
CD 4	232.4 (77.7–589.1)	377.8 (166.0–683.1)	NS
CD8	118.3 (28.5–392.9)	239.1 (48.8–427.7)	NS
CD14	28.8 (11.2–56.2)	28.9 (13.5–62.3)	NS
AA1	40.0 (27.8–81.2)	76.3 (28.2–156.3)	0.009
Neutrophil Elastase	22.4 (4.9–42.3)	65.0 (12.1–376.1)	0.005
EG2	4.5 (0.0–11.2)	5.2 (1.5–77.4)	NS

*Values are medians, with interquartile ranges shown in parentheses. Cells are expressed as cells/mm² submucosa. The cytokines, chemokines, adhesion molecules and NF- κ B are expressed as % of the total number of vessels revealed by staining with the pan endothelial mAb EN4.

†Wilcoxon's matched paired sign rank test.

The mAb EN4 clearly picked out the endothelium of the microvasculature. In the microvascular epithelium, P-selectin, E-selectin, and ICAM-1 were expressed constitutively in biopsies obtained from subjects exposed to air, but only a very low level of VCAM-1 staining was observed. After ozone exposure there was significant increase of the proportion of blood vessels that expressed E-selectin ($p = 0.01$), ICAM-1 ($p = 0.005$), and activated form of NF- κ B ($p = 0.05$) (Tabl. 1b). No significant differences were observed in the level of expression of P-selectin, and VCAM-1 between the two exposures. After ozone exposure there was a significant increase in the expression of proinflammatory cytokines TNF- α ($p = 0.02$), and IL-1 β (0.009) on the blood vessels in the microvasculature but no significant differences in the expression of IL-6 and GM-CSF between the two exposures. Regarding the chemokines examined in our biopsies, there was a significant increase of the proportion of blood vessels that expressed IL-8 ($p = 0.02$) but no change in the expression of RANTES was seen. In addition significant correlations were seen between IL-8 expression and the number of neutrophils in the submucosa ($r = 0.84$; $p = 0.004$), TNF- α and ICAM-1 expression ($r = 0.75$; $p = 0.01$), TNF- α and NF- κ B expression ($r = 0.79$; $p = 0.006$) and ICAM-1 and NF- κ B expression ($r = 0.67$; $p = 0.03$) all after exposure to 400 ppb ozone. These results suggest that activation of NF- κ B and up regulation of adhesion molecules and IL-8 as well as some other pro-inflammatory cytokines contribute to the neutrophil recruitment and inflammation after ozone inhalation in normal subjects.

Table Ib

*Immunohistochemistry data (adhesion molecules and cytokines)**

mAb	Air	Ozone	p-Value†
P-selectin, %	23.5 (12.1-42.5)	49.2 (19.3-78.5)	NS
E-selectin, %	19.3 (10.2-35.9)	44.1 (14.8-64.5)	0.01
ICAM-1, %	31.8 (10.1-46.1)	54.2 (24.0-88.3)	0.005
VCAM-1, %	7.6 (0.0-23.9)	12.1 (0.0-32.5)	NS
RANTES	0.0 (0.0-0.0)	0.0 (0.0-2.1)	NS
IL-8, %	0.0 (0.0-3.7)	2.5 (0.0-7.3)	0.02
IL-6, %	0.0 (0.0-5.4)	1.1 (0.0-12.6)	NS
IL-1 β , %	8.7 (0.0-24.2)	29.3 (3.7-53.5)	0.009
TNF- α , %	0.0 (0.0-3.0)	2.3 (0.0-7.3)	0.02
GM-CSF, %	0.0 (0.0-1.0)	0.5 (0.0-8.4)	NS
NF- κ B, %	0.0 (0.0-5.3)	1.5 (0.0-16.4)	0.05

*Values are medians, with interquartile ranges shown in parentheses. Cells are expressed as cells/mm² submucosa. The cytokines, chemokines, adhesion molecules and NF- κ B are expressed as % of the total number of vessels revealed by staining with the pan endothelial mAb EN4.

†Wilcoxon's matched paired sign rank test.

Discussion

The purpose of our study was to examine the events involved in ozone-induced inflammatory cell recruitment. We specifically examined the effects of 400 ppb ozone on nasal mucosa in normals at rest for 2 h. The level and duration of ozone exposure were selected because they are the same as those that have induced neutrophil influx into the nasal tissue of normal subjects as reported by other authors [2, 3, 12, 13]. Although the level of ozone used was relatively high (400 ppb) exposure was only for 2 h and occurred without exercise, unlike a study of normal subjects who underwent heavy exercise [12]. Each subject underwent both an ozone and filtered air exposure, which was followed by nasal biopsy 6 h after the exposure. Previous studies have shown that short-term exposure to ozone at a concentration of 80-400 ppb ozone induces neutrophilic inflammation that peaks 6 and 10 h after exposure [12, 14, 15] which was why we chose to take biopsies 6 h post exposure to ozone. We found a significant increase in the number of neutrophils and mast-cells in the sub mucosa after ozone exposure.

Ozone has been shown to induce inflammatory changes in upper airways of both normal and allergic subjects. Graham and Koren [2] have demonstrated that ozone caused increased neutrophil content of the nasal lavage fluid

(NLF) of nonallergic subjects, indicating that ozone also induces nasal inflammation. Fischer and colleagues [5] 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. There are many reports that ozone-induced neutrophilia in the BAL is associated with increased levels of IL-8, IL-6, GM-CSF and other inflammatory markers [12, 14, 15]. We also found increased expression of IL-8 on the microvasculature. Furthermore, we found a significant correlation between the expression of IL-8 on the blood vessels and the number of neutrophils in the submucosa indicating that IL-8 plays an important role in neutrophil recruitment. IL-8 is a potent neutrophilic chemoattractant [16] that induces shape change [17], the shedding of L-selectin [18], and the up regulation of adhesion molecules on the neutrophil cell surface [19], events essential for cell recruitment from the circulation. IL-8 activates neutrophil degranulation [20] and respiratory burst [21] and is therefore likely to play a role in neutrophilic inflammation. Other cell types also respond to this chemokine [22–26]. There are reports that IL-8 and RANTES are chemoattractant for mast cells which number was significantly higher in the submucosa of the patients when exposed to 400 ppb ozone. The role of the mast cell in the ozone-induced inflammation remains uncertain but the injury of epithelial cells and mast cells results in increased permeation or accumulation of plasma proteins within the interstitium or airspace [27, 28], triggers further inflammation [12], and stimulates neural afferents in the airways. Although there is evidence that IL-8 is a potent eosinophil chemoattractant [29] we did not see a significant increase in the number of eosinophils in the submucosa. Additionally, there was no significant change in the expression of RANTES and VCAM-1 on the microvasculature indicating that ozone does not induce eosinophil recruitment in normal subjects. Boscom and co-workers [3] have reported that normal subjects did not have an ozone-induced increase in eosinophils to the nasal mucosa, as did persons with allergic rhinitis, indicating that eosinophil influx to the nasal airway is a unique response of allergic subjects to ozone exposure.

In our study we found increased expression of E-selectin and ICAM-1 6h after exposure to ozone. Up regulation of ICAM-1 on the vascular endothelium and increase in the number of CD11a/CD18⁺ and CD11b/CD18⁺ neutrophils in BAL fluid by ozone at 18 h after short-term exposure to 200 ppb ozone have been recently reported, suggesting that ICAM-1 plays a role in the neutrophil migration after ozone exposure [30, 31].

Although we did not find increased expression of P-selectin it is likely to be one of the earliest stages of leukocyte recruitment since this adhesion molecule is involved in leukocyte rolling [32, 33]. P-selectin exists as a preformed molecule and its storage in Weibel-Palade bodies of endothelial cells allows its mobilization to the surface in less than 5 min of endothelial activation.

P-selectin is soon internalized by the endothelial cells and is not detectable after 2h. This could be the reason why we did not see any up regulation of P-selectin since our biopsies were taken 6h post exposure. Whereas the expression of P-selectin relates to the transport of a preformed adhesion molecule, the expression of E-selectin involves post-stimulation transcription [34]. Surface expression of E-selectin has been reported to peak 4 h after stimulation, with a return to basal levels of expression within 24h [35]. ICAM-1 and E-selectin expression is induced when the cells are activated by IL-1 β and TNF- α [36, 37]. These cytokines activate the transcription of E-selectin and ICAM-1 via the release of an activated form of NF- κ B, resulting in peak cell surface expression at 4–6h. Products of the genes that are regulated by NF- κ B also cause the activation of NF- κ B [38]. The proinflammatory cytokines IL-1 β and TNF- α both activate and are activated by NF- κ B. This positive regulatory loop may amplify and perpetuate local inflammatory responses [38]. We found significantly increased expression of TNF- α , IL-1 β and NF- κ B on the microvasculature. Ozone itself or via its intermediate product H₂O₂ can trigger a reaction that leads to the degradation of the bound I κ B protein in the epithelial cells. Loss of the inhibitory I κ B 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 κ B sites in the promoter and enhancer genes. NF- κ B is implicated in the activation of several gene families encoding proinflammatory cytokines (IL-1 β , TNF- α , IL-6, IL-2, GM-CSF, MC-SF, GC-SF), chemokines (IL-8, MIP-1 α , MCP-1, Gro- α , - β and γ and eotaxin), inflammatory enzymes (iNOS, inducible cyclooxygenase-2, 5-lipoxygenase, cytosolic phospholipase A₂), adhesion molecules (ICAM-1, VCAM-1, E-selectin) and receptors (IL-2R, T-cell receptor) (38-40). The activation of NF- κ B leads to a coordinated stimulation of the expression of the genes for E-selectin, ICAM-1, IL-8, TNF- α and IL-1 β in the endothelial cells resulting in the recruitment and activation of neutrophils. We found a significant correlation between TNF- α and ICAM-1 expression ($r = 0.75$; $p = 0.01$), TNF- α and NF- κ B expression ($r = 0.79$; $p = 0.006$) and ICAM-1 and NF- κ B expression ($r = 0.67$; $p = 0.03$) all after exposure to 400 ppb ozone.

Short-term exposure to 400 ppb ozone induces infiltration of neutrophils into the nasal mucosa 6 h post exposure as a result of increased expression of IL-8 and adhesion molecules (ICAM-1 and E-selectin). Since both TNF- α and IL-1 β can induce NF- κ B (38), it is tempting to postulate that ozone may induce TNF- α and IL-1 β which in turn increase NF- κ B-binding to cause IL-8, ICAM-1 and E-selectin m-RNA up regulation

Conclusion

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.

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 a result of increased synthesis and release of inflammatory cytokines from the airway epithelial cells. Exposure of normal subjects to ozone increases the expression of proinflammatory cytokines resulting in up regulation of IL-8 and adhesion molecules via activation of NF- κ B, leading to neutrophil infiltration in the nasal mucosa.

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ЕФЕКТ НА ОЗОНОТ ВРЗ НАЗАЛНАТА МУКОЗА (ЕНДОТЕЛНИ КЛЕТКИ)

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Апстракт: Човековиот организам кога ќе се експонира на озон развива воспалителна реакција на назалната мукоза која се карактеризира со неутрофилна инфилтрација. Со цел да ги испитаме механизмите на инфламаторната реакција во назалната мукоза, десет здрави непущачи ги експониравме на 400 ппб озон или филтриран воздух во траење од 2 часа во два различни дена. Шест часа по експозицијата на озон или филтриран воздух, кај пациентите се изведени назални биопсии. Потоа, тие беа импрегнирани во гликол метакрилат и имунохистохемиски боени со цел да се идентификуваат и избројат воспалителните клетки, како што се неутрофилите, маст-клетките, вкупните Т-лимфоцити (CD3), Т-клеточните супсетови CD8 и CD4, макрофагите, еозинофилите, адхезивните молекули (P-selectin, E-selectin, ICAM-1, VCAM-1), цитокините (TNF- α , IL-1 β , GM-CSF, IL-6), хемокините (IL-8 and RANTES), како и нуклеарниот фактор NF- κ B.

Во назалните биопсии немаше сигнификантна разлика во бројот на вкупните Т-лимфоцити и Т-клеточните супсетови, еозинофилите, макрофагите или процентот на крвните садови коишто експримираа P-selectin, VCAM-1, GM-CSF, IL-6 и RANTES. Бројот на неутрофилите и маст-клетките во супмукозата беа сигнификантно поголеми кај пациентите по експозицијата со озон ($p = 0.009$ и $p = 0.005$ соодветно). Процентот на крвните садови коишто

експримираа E-selectin ($p = 0.01$), ICAM-1 ($p = 0.005$), IL-8 ($p = 0.02$), TNF- α ($p = 0.02$), IL-1 β ($p = 0.009$), и NF- κ B ($p = 0.05$) беше сигнификантно поголем по експонирањето на озон во споредба со експозицијата на филтриран воздух.

Експозицијата на здравите испитаници на озон ја зголемува експресијата на проинфламаторни цитокини резултирајќи со зголемена регулација на IL-8 и адхезиони молекули преку активација на NF- κ B што води до неутрофилна инфилтрација на назална мукоза.

Клучни зборови: озон, неутрофили, ICAM-1, цитокини, назална мукоза.

Кретенки: ICAM-1; интерцелуларна адхезивна молекула 1
VCAM-1; васкуларно-клеточна адхезивна молекула 1
NF- κ B; нуклеарен фактор - κ B
TNF- α ; тумор некротизирачки фактор алфа
IL-1 β ; интерлеукин 1 бета
IL-6; интерлеукин 6
IL-8; интерлеукин 8
RANTES; Regulated on Activation, Normal T Expressed and Secreted
GM-CSF; гранулоцитно-моноцитен стимулирачки фактор на колониите

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