NADPH Quinone Oxidoreductase 1 Regulates Host Susceptibility to Ozone via Isoprostane Generation

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Running title: NQO1 regulates host susceptibility to ozone via A_2-isoprostane
Background: NQO1 regulates pulmonary susceptibility to ozone.

Results: In NQO1-null mice, ozone exposure generates precursors of A_2-isoprostane in the lung. A_2-isoprostane suppresses ozone-induced IL-8 expression, inhibits NF-κB, and modifies Cys179 in IKK.

Conclusions: A_2-isoprostane inhibits ozone-induced NF-κB activation via IKK inhibition.

Significance: This molecular mechanism explains the paradoxical observation that loss of NQO1 protects from ozone toxicity.

Summary

NADPH quinone oxidoreductase 1 (NQO1) is recognized as a major susceptibility gene for ozone-induced pulmonary toxicity. In the absence of NQO1, as can occur by genetic mutation, the human airway is protected from harmful effects of ozone. We recently reported that NQO1-null mice are protected from airway hyperresponsiveness and pulmonary inflammation following ozone exposure. Yet, NQO1 regenerates intracellular antioxidants and therefore should protect the individual from oxidative stress. To explain this paradox, we tested whether in the absence of NQO1, ozone exposure results in increased generation of A_2-isoprostane, a cyclopentenone isoprostane that blunts inflammation. Using GC/MS, we found that NQO1-null mice had greater lung tissue levels of D_2- and E_2-isoprostanes, the precursors of J_2,- A_2-isoprostanes, both at baseline and following ozone exposure compared to congenic wild-type mice. We confirmed in primary cultures of normal human bronchial epithelial cells, that A_2-isoprostane inhibited ozone-induced NF-κB activation and IL-8 regulation. Furthermore, we determined that A_2-isoprostane covalently modified the active Cys179 domain in Inhibitory κB Kinase in the presence of ozone in vitro, thus establishing the biochemical basis for A_2-isoprostane inhibition of NF-κB. Our results demonstrate that host factors may regulate pulmonary susceptibility to ozone by regulating the generation of A_2-isoprostanes in the lung. These observations provide the biochemical basis for the epidemiologic observation that NQO1 regulates pulmonary susceptibility to ozone.
Introduction

Ozone induces airflow obstruction and pulmonary inflammation

In epidemiologic studies, ozone levels directly correlate with emergency department visits for asthma, school absences, and hospitalization rates (1-3). Ozone inhalation causes several pulmonary responses including increased cough, chest pain, decreased FEV\textsubscript{1}, airway hyperreactivity (AHR) (4), and increased airway inflammation, specifically neutrophilic inflammation (1,5). Ozone triggers these physiologic sequelae via the generation of reactive oxygen species that act as potent intracellular signals.

Ozone generates reactive oxygen species and activates transcription factors leading to pulmonary inflammation

Ozone generates lipid ozonation products (LOP), including aldehydes, hydroxyhydroperoxides and hydrogen peroxide (6) in the airway. LOPs and downstream reactive oxygen species activate transcription factors including nuclear factor kappa from B cells (NF-κB) (7,8). NF-κB activation is essential for pulmonary inflammation following ozone exposure (9,10). Ozone triggers NF-κB by activating the IkB kinase (IKK) complex resulting in phosphorylation of IkB-α which is degraded resulting in the release and nuclear translocation of the NF-κB heterodimer, which often includes p65/ RelA (11). Ozone exposure of human airway epithelial cells in vitro results in increased production of inflammatory mediators including granulocyte macrophage-colony stimulating factor, TNF-α, and IL-8 (12-14).

NADPH Quinone Oxidoreductase 1 is a host factor for pulmonary susceptibility to ozone

An NADPH quinone oxidoreductase 1 (NQO1) polymorphism (15,16) has been reported in epidemiologic studies to affect pulmonary susceptibility to ozone. NQO1 is highly expressed in airway epithelia (17), and catalyzes the reduction of quinones such as ubiquinone and α-tocopherone to regenerate antioxidant capacity and prevent lipid peroxidation of cellular membranes (18) (19). One NQO1 single nucleotide polymorphism, C609T (Ser187 variant) accelerates degradation of NQO1 resulting in functional loss of the enzyme. Importantly, this NQO1 polymorphism, 609TT (187Ser/Ser), has a protective effect against asthma in children with GSTM1-null genotype and high lifetime ozone exposure (20), and protects subjects from airway obstruction in high ozone environments (16,20,21).

Consistent with this epidemiologic data, we reported that compared to congenic C57BL/6 wild-type mice, NQO1-null mice were protected from ozone-induced airway hyperresponsiveness and airway inflammation. Paradoxically, following ozone exposure, NQO1-null mice produced significantly less F\textsubscript{2}-isoprostane, a non-enzymatic peroxidation product of arachidonic acid that is a stable biomarker of oxidant stress (22). These results were unexpected since absence of NQO1 should increase oxidative stress post-ozone. Therefore, we investigated why there was a difference in F\textsubscript{2}-isoprostane production in wild-type vs. NQO1-null mice, and investigated whether the difference in isoprostane production affected pulmonary responses to ozone.

Isoprostanes and host response to ozone

Isoprostanes (IsoPs) are formed by the non-enzymatic peroxidation of arachidonic acid. They are named according to the prostaglandin isomer closest in structure. F\textsubscript{2}-IsoPs (isomers of PGF\textsubscript{2α}) are chemically stable molecules that are accepted as a “gold standard” biomarker of endogenous oxidative stress. IsoPs with an E\textsubscript{2} or D\textsubscript{2} ring are also generated in vivo (23) depending upon the reduction-oxidation (redox) status of the cell (24) and spontaneously undergo dehydration to form the cyclopentenone IsoPs, known as A\textsubscript{2}- and J\textsubscript{2}-IsoPs respectively. A\textsubscript{2}-/J\textsubscript{2}-IsoPs contain a highly reactive α,β-unsaturated carbonyl group on the cyclopentenone ring that readily addsucts thiols via Michael addition...
which allows these molecules to exert potent biological activities. For example, $A_2$-IsoPs have been reported to inhibit activation of NF-$\kappa$B, suggesting that they have an anti-inflammatory function. (25) (26).

In this report we tested whether NQO1-null mice generated greater levels of $E_2/D_2$-IsoPs, the $A_2/J_2$-precursors, following ozone exposure compared to control mice. We also tested whether $A_2$-isoPs inhibited ozone-triggered NF-$\kappa$B activation and IL-8 expression in normal human bronchial epithelial cells (NHBE). Our results suggest a novel biochemical mechanism to explain the protection afforded by loss of NQO1 against ozone-induced pulmonary inflammation.
Experimental procedures

Cell Culture

The primary normal human bronchial epithelial (NHBE) cells were harvested from human tracheobronchial tissues from donors obtained from the Lung Transplant Program and the Department of Pathology, Duke University Medical Center. The protocol was approved by the Institutional Review Board for Clinical Investigations, Duke University Medical Center. Cells were plated as previously described on 6- or 12-well Transwell Clear chambers (Corning, Corning, NY), in a serum-free growth factor supplemented media with all-trans-retinoic acid (RA) (27, 28) and cultured in air-liquid interface (ALI) culture conditions. Experiments were performed on days 10-14 after the change of the culture condition to ALI. NHBE cells were changed to media supplemented with retinoic acid, for 24 h before ozone exposure. Cells were pre-incubated with A2-isoPs (provided by Dr. Ginger Milne or Cayman Biochemical, 50nM or 2.5 μM) or the equivalent volume of control vehicle (N2-purged ethanol) and then co-incubated with ozone or filtered air (FA) (see ozone exposure section below).

Mouse Models

C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). A breeding colony was established at Duke University from breeding pairs of NQO1-null mice (on a C57BL/6J background) obtained from Dr. Frank Gonzalez at the National Cancer Institute (Bethesda, MD). Male C57BL/6J or NQO1-null mice were used at 6 to 8 weeks of age. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and were performed in accordance with the standards established by the U.S. Animal Welfare Act.

Ozone Exposure

Mice were exposed to either ozone (1 ppm) or filtered air for 3 h (as previously reported) (22). The ozone concentration in the chamber was continuously monitored with an ozone ultraviolet light photometer (Dasibi model 1003AH; Dasibi Environmental Corp., Glendale, CA). Mouse lungs were harvested 24 h post-ozone or filtered air exposure, snap frozen and stored at -80°C until lipid extraction for E2-, D2-isoprostane analysis.

NHBE cells were exposed to either FA or 0.4 ppm ozone from 30 min to five hours in exposure chambers; each gas was provided at 20 L/minute, balanced with 5% CO2, and at 88% relative humidity. Immediately after the exposure, cells were processed for isolation of total RNA, cell lysate protein, or basolateral media collection. By LDH assay, ozone does not cause cytotoxicity at this concentration or duration of exposure. ELISA [LEGEND MAX Kit for human IL8, BioLegend, San Diego, CA] was performed for IL-8 quantitation in basolateral media diluted 1:5 or 1:10 as per the manufacturer’s instructions.

Analysis of E2-/D2-isoprostane

D2-/E2-isoprostanes were analyzed by gas chromatography-negative ion chemical ionization-mass spectrometry (GC/NICI/MS) using a modification of methods described previously for the analysis of PGE2 and PGD2, (29). Briefly, 1.5 ng of [2H4] PGE2 internal standard is initially added to a biological fluid and adjusted to pH 3 with 1 M HCl. The sample is then applied to a C-18 Sep-Pak cartridge compounds are eluted with 10 ml of ethyl acetate and evaporated to dryness under nitrogen. Compounds are subsequently derivatized by treatment with a 2% solution of aqueous methoxyamine.HCl, extracted with ethyl acetate and the organic layer evaporated under nitrogen. Compounds are converted to a pentafluorobenzyl (PFB) ester, dried under nitrogen and the residue reconstituted in 30 μl of chloroform and 20 μl of methanol and chromatographed on a silica TLC plate to 13 cm in a solvent system of ethyl acetate:methanol (98:2, v/v). The methyl ester of PGF2α and the 0-methyloxime, PFB ester derivative of PGD2
(approximately 5 µg each) are chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The R_F of PGF\textsubscript{2α} methyl ester in this solvent system is ~0.25, and the R_F of the 0-methyloxime, PFB ester derivative of PGF\textsubscript{2} is ~0.60. Compounds migrating in the region 1 cm above the PGF\textsubscript{2} standard to 0.5 cm below the PGF\textsubscript{2} standard are scraped from the TLC plate, extracted with 1 ml of ethyl acetate, and dried under nitrogen. Following TLC purification, compounds are converted to trimethylsilyl (TMS) ether derivatives and dried under nitrogen. The residue is redissolved for GC/MS analysis in 10 µL undecane that has been stored over a bed of calcium hydride.

GC/NICI-MS is carried out on an Agilent 5973 Inert Mass Selective Detector that is coupled with an Agilent 6890n Network GC system that is interfaced with an Agilent computer. The GC is performed using a 15 m, 0.25 mm film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190° to 300°C at 20°C per minute. The major ion generated in the NICI mass spectrum of the PFB ester, TMS ether derivatives of the E\textsubscript{2}/D\textsubscript{2}-IsoPs is m/z 524 while the [\textsuperscript{2}H\textsubscript{4}]-PGE\textsubscript{2} internal standard has a m/z of 528. Levels of endogenous eicosanoids in the biological samples are calculated from the ratio of intensities of the [\textsuperscript{2}H\textsubscript{0}]- and [\textsuperscript{2}H\textsubscript{4}]-ions.

**Total RNA collection and Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)**

RNA was isolated from NHBE cells using Trizol (Invitrogen) according to the manufacturer’s instructions and IL-8 gene expression was analyzed by quantitative real-time RT-PCR on an SDS 7300 machine (Applied Biosystems) in a 25µl reaction which contained 1× TaqMan gene expression assay (mixture of PCR primer set specific for IL-8 and a FAM dye-labeled TaqMan MGB probe), using universal amplification conditions. Amplification reaction of 18s rRNA control contained 0.2µM forward primer, 0.2µM reverse primer, and 0.8µM VIC dye-labeled probe. Each sample was amplified in duplicate reactions for both the gene of interest and 18s rRNA control. The relative gene expression level was calculated by the ∆ΔCt method which represents the fold difference in gene expression corrected for 18s rRNA control expression and normalized to the control treated sample (28,30).

**Isolation of cellular proteins**

Ozone-exposed cells were rinsed with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed, for 5 min on ice, in Cell Lysis Buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycercophosphate, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 µg/ml leupeptin, and 1mM phenylmethylsulfonyl fluoride] (Cell Signaling Technology, Beverly, MA) containing both 1x complete protease and 1x phosphatase inhibitor cocktails (Sigma). Cultures were then scraped and disrupted mechanically by pipetting and by sonication. Samples were centrifuged at 14,000 rpm for 20 min at 4°C using a tabletop microcentrifuge. Total cellular protein concentrations in each supernatant were determined by Bio-Rad DC Protein Assay (Bio-rad, Hercules, CA).

**Western blot analysis**

To determine the activation of the NF-κB pathway in NHBE cells exposed to ozone vs. filtered air, the phosphorylation of subunit p65 of NF-κB was evaluated. Equal amounts of total proteins (60-100 µg/lane) were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel, and then transferred onto nitrocellulose membranes for Western analysis. Nonspecific binding was blocked with 5% milk powder in TBST [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20] for 1 hour at room temperature, followed by incubation with primary rabbit polyclonal antibody specific to phospho-p65 (Ser-536) (diluted 1:1000, from Cell Signaling Technology, Beverly, MA) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG
HRP-linked antibody, 1:2000, Cell Signaling Technology, Beverly, MA) for 1h at room temperature. After two 20-min washes in TBST, immunoreactive protein complexes were detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). To confirm equal loading, blots were rehybridized with mouse monoclonal antibody specific to β-actin (1:5000, Sigma-Aldrich, St. Louis, MO).

**IKK activity assay**

The IκB kinase (IKK) enzyme activity was evaluated in the proteins isolated from OZ or FA-exposed cells. The IKK enzyme was selectively immunoprecipitated from 500 μg of total cellular protein, in a 500 μl reaction volume of cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1mM phenylmethylsulfonyl fluoride containing both 1x complete protease and 1x phosphatase inhibitor cocktails] using rabbit polyclonal antibodies that recognized IKKα (1:250) and IKKβ (1:50) (Cell Signaling Technology, Beverly, MA) or rabbit IgG as control. Following overnight immunoprecipitation at 4˚C, the immune complexes were then precipitated with 25 μl volume of magnetic Dynabeads protein G (4˚C, 4 h) (Invitrogen). Beads were washed twice with lysis buffer and then twice with kinase reaction buffer (25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl₂) (Cell Signaling Technology, Beverly, MA). The IKK activity assay was initiated by the addition of kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl₂) (Cell Signaling Technology, Beverly, MA). The IKK activity assay was initiated by the addition of kinase buffer containing 200 μM ATP, and 1 μg GST-IkBα as a substrate for the IKK enzyme activity. The kinase reactions were incubated at 30˚C for 30 min and were then stopped by addition of SDS sample loading buffer. The reaction mixture was boiled for 5 min and aliquots resolved by 10% SDS-PAGE, followed by Western analysis using antibodies to phospho-IkBα (ser32/36) (mouse monoclonal, 1:1000 dilution, Cell Signaling Technology, Beverly, MA) and horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse IgG HRP-linked antibody, 1:2000, GE Healthcare Bio-Sciences Corp, Piscataway, NJ). After two 20-min washes in TBST, immunoreactive protein complexes were detected by enhanced chemiluminescence. IKK activity was determined by the intensity of phospho-GST-IkBα (ser32/36) signals on the autoradiogram. The blots were rehybridized with antibodies against IKKβ (rabbit polyclonal antibody, 1:1000 dilution, Cell Signaling Technology, Beverly, MA) and immunoreactive protein complexes were detected by enhanced chemiluminescence. The band densities of phosphorylated IκB-α were normalized to the corresponding immunoprecipitated IKKβ band densities.

**IKK Cys179 Peptide In Vitro Modification Analysis by Mass Spectrometry**

**Peptide Preparation** - To investigate the modification mechanism of reactive Cys-179 residue of IKK-β peptide with isoprostanes, two synthetic 27-aa peptides encompassing E172-K198 of the primary sequence of human IKK-β containing Cys-179 residue (WT, 3009.500 MW) and Cys-179-Ala (MUT, 2977.528 MW) were obtained commercially (Invitrogen, Carlsbad, CA). These peptides were dissolved in nitrogen purged N,N-Dimethylformamide (DMF; 5 µg/µl) and stored at -80°C. An aliquot of 10-µl (50 µg/166µM) was further diluted in PBS to obtain 100 µl reaction volume in a shallow dish, and exposed to ozone (0.4 ppm,30 min) or filtered air in the presence or absence of A2-isoprostane (166 µM), or as a control, F2-isoprostane (166 µM). Peptides under each of the conditions were frozen in aliquots at 1 mg/mL at -80°C for mass spectrometry analysis in the Duke Proteomics Core Facility. Upon thawing, 5 µL of each peptide was transferred into a separate tube and 15 µL of 0.1% trifluoroacetic acid (TFA) was added to each. Millipore C18 ZipTips® were used according to
the manufacturer’s protocol to desalt 5 µg of each peptide sample. The samples were vacuum-dried and dissolved in 100 µL of 2/98/0.1 v/v/v MeCN/H₂O/TFA.

**LC/MS Data collection** – Each of the six peptide exposure conditions were analyzed using a nanoAcquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters Corp, Milford, MA). Approximately 50 ng of peptide material in 1 µl was first trapped at 5 µl/min for 3 minutes in 99.9% water with 0.1% v/v formic acid on a 20 µm × 180 mm Symmetry C18 column. Separations were then performed on a 75 µm × 250 mm column with 1.7 µm C18 BEH particles (Waters) using a 30-min gradient of 5 to 40% acetonitrile with 0.1% formic acid at a flow rate of 0.4 µl/min and 55°C column temperature. The samples were then subjected to MS/MS acquisition in data-dependent analysis (DDA) mode, using a 0.9 sec MS scan followed by MS/MS acquisition on the top 3 ions with charge greater than 1. MS/MS scans for each ion used an isolation window of approximately 3 Da, a maximum of 3 seconds per precursor, and dynamic exclusion for 120 seconds within 1.2 Da.

**LC-MS Data Processing** – The resulting raw data files were processed using Mascot distiller and processed spectra were submitted to Mascot v2.2 (Matrix Sciences, Inc) search engine. The data were searched against the current SwissProt database with *Homo Sapiens* taxonomy, semitryptic enzyme specificity, precursor ion mass tolerance of 10 ppm, and product ion tolerance of 0.04 Da. Dynamic peptide modifications allowed were single, double or triple oxidation on cysteine, cysteine mutation to alanine (-31.972 Da), and oxidized (334.2144 Da) or reduced (336.230 Da) isoprostane modification of cysteine. Database search results for all conditions have been made publicly available as a Scaffold file (.sf3, www.proteomesoftware.com), at the following link:

https://discovery.genome.duke.edu/express/resourc es/2473/2473_122110.sf3

**Statistical Analysis**

Except for the analysis of A₂-isoP modification of the IKK peptide, all other experimental results were expressed as mean±SEM, and differences between mean values were analyzed by one way analysis of variance (ANOVA) with post-hoc analysis by the Wilcoxon Rank sum test (Statistix 8, Analytical Software, Tallahassee FL). P values < 0.05 were considered statistically significant.
Results

NQO1-null mice had greater levels of E$_2$/D$_2$-IsoPs (A$_2$/J$_2$-IsoP precursors) both at baseline and post-ozone exposure compared to wild-type mice.

We previously reported that ozone exposure increases BAL F$_2$-isoP levels, a marker of oxidative injury, in wild type mice; but not in NQO1-null mice (22). In this report, we measured by gas chromatography-negative ion chemical ionization -mass spectrometry (GC-NICI-MS) murine lung tissue levels of E$_2$/D$_2$-IsoPs, in wildtype and NQO1-null mice after filtered air or ozone exposure. There was increased generation of E$_2$/D$_2$-IsoPs in NQO1-null mice compared to wild-type mice both after filtered air and after ozone exposure. These results demonstrate that absence of NQO1 favored the generation of E$_2$/D$_2$-IsoP in murine lung tissue.

A$_2$-IsoP blocked ozone-induced IL-8 mRNA expression in human airway epithelial cells.

To determine the effect of ozone exposure on pro-inflammatory cytokine expression in human airway epithelial cells, we used qRT-PCR to measure IL-8 mRNA expression in normal human bronchial epithelial cells (NHBE) cultured at air liquid interface and exposed to filtered air or ozone (0.4 ppm) over time (ranging from 30 min to 5 hours) (Figure 2A). IL-8 mRNA levels were normalized to 18S rRNA levels and expressed as a percentage of the 30 min filtered air control treatment condition. Ozone exposure increased IL-8 expression in a time-dependent manner with a significant increase in IL-8 expression by 1 hour of ozone exposure, and progressive increase in IL-8 expression up to 5 h.

To explore the anti-inflammatory effect of A$_2$-IsoP in ozone-exposed NHBE, we examined the effect of A$_2$-IsoP on ozone-induced IL-8 gene expression (Figure 2B-E). We first pretreated epithelial cells with two different concentrations of A$_2$-IsoP (50 nM & 2.5 μM) for 1 hour and then exposed cells to ozone or filtered air for 2 or 5 hours. IL-8 mRNA expression was significantly up-regulated by ozone exposure both at 2 h (Figure 2B and 2C) and 5 h (Figure 2D and 2E). A$_2$-IsoP blocked ozone-upregulation of IL-8 at both concentrations and at both time points.

A$_2$-IsoP inhibited the ozone-induced increase in IL-8 protein released from human airway epithelial cells.

We tested whether A$_2$-IsoP inhibition of ozone-upregulated IL-8 mRNA levels affected protein expression of IL-8. NHBE were exposed to filtered air or ozone (0.4 ppm) for 5 h in the presence or absence of A$_2$-IsoP (2.5 μM) and basolateral media was collected for ELISA determination of IL-8 (Figure 3). Ozone significantly increased IL-8 protein levels in NHBE from two different donors and exposure to A$_2$-IsoP (2.5 μM) blocked this release.

A$_2$-IsoP abrogated ozone-induced NF-κB activation in normal human bronchial epithelial cells.

NF-κB is a major transcription factor activated by ozone and required for upregulation of IL-8 expression (7). We examined the effect of A$_2$-IsoP on ozone-activation of the NF-κB pathway in NHBE using two complementary methods. First, we examined activation/phosphorylation of one component of the NF-κB heterodimer, p65 by western analysis (Figure 4), and second, we evaluated the activity of the IKK complex by using the same cell lysates to immunoprecipitate IKKα and IKKβ and then perform a kinase assay to evaluate IKK activity-phosphorylation of substrate IkBα detected by western analysis (Figure 5).

Following NHBE exposure to filtered air or ozone (0.4 ppm, 30, 60 or 120 min), total protein lysates were evaluated by western analysis for p65 phosphorylation (Figure 4A). The results demonstrated a time-dependent increase in phosphorylation of p65 that was evident at 30 and 60 min, but not present at 120 min of ozone exposure (Figures 4A and 4B). No changes in phosphorylation of p65 were
observed in cells exposed to filtered air, and ozone exposure did not upregulate total p65 concentrations in cell lysates.

We next investigated the effect of A2-IsoP (50 nM & 2.5 µM) on ozone-induced NF-kB activation by measuring the phosphorylation of p65 both at 30 and 60 min of ozone exposure (Figures 4C-F). Ozone-induced p65 phosphorylation in NHBE cells was significantly attenuated by co-incubation with A2-IsoP at both concentrations and at both 30 min (Figures 4C and 4D) and 60 min (Figures 4E and 4F) of ozone exposure. A2-IsoP alone with filtered air exposures did not affect p65 phosphorylation at either A2-IsoP concentration or time point.

To further confirm the activation of the NF-kB pathway, we analyzed the effect of ozone on IKK activity. IKKα and IKKβ, are the catalytic moieties of the IKK complex, and their activity is required for IκBα phosphorylation, degradation, and release of NF-kB for its translocation to the nucleus. To test activation of the IKK complex by ozone and potential inhibition by A2-IsoP, IKKα and IKKβ were immunoprecipitated from total protein lysates from cells exposed to filtered air or ozone (0.4 ppm, 30 and 60 min) and their activities were determined by in vitro kinase assays with a fusion protein GST-IκBα as substrate. This substrate contains the specific phosphorylation sites Ser-32 and Ser-36 and eliminates the possibility of nonspecific phosphorylation at the C terminus of full-length IκBα protein. Specificity was ensured by using normal rabbit IgG as a negative control, which failed to precipitate IKKα or IKKβ. In agreement with results demonstrating phosphorylation of p65, ozone exposure increased IKK activity at 30 and 60 min, but not 120 min in NHBE (Figure 5A). There was no change in total IKK expression following ozone exposure.

We then tested IKK activity in the presence or absence of A2-IsoP following filtered air or ozone exposure (0.4 ppm, 30 and 60 min) of NHBE. A2-IsoP at both 50 nM and 2.5 µM significantly inhibited ozone-induced IKK activity at both 30 min (Figures 5B and 5C) and 60 min (Figures 5D and 5E). Changes in activity were not due to loss of IKK as there was no change in IKK expression levels in NHBE under any treatment condition. These data suggest that co-incubation of A2-IsoP inhibited IKK activity and subsequent phosphorylation of p65 thereby preventing NF-κB nuclear translocation and transcriptional upregulation of IL-8 in ozone-exposed NHBE cells.

A2-IsoP covalently modified the Cys-179 sulphydryl in the active loop of IKK, an inactivating post-translational modification.

The Cys-179 residue in the active loop of IKK is susceptible to oxidation and inactivation (31). To determine whether it is possible that A2-IsoP inactivates IKK by oxidation/ Michael adduction of the sulphydryl group, a 27 aa synthetic IKKβ peptide E172K198 encompassing the Cys 179 residue, was exposed to filtered air or ozone (0.4 ppm, 30 min) in the presence or absence of equimolar amounts of A2-IsoP or F2-IsoP. Using nanoscale LC-MS/MS analysis, we evaluated whether any of these treatment conditions chemically modified the Cys179 residue of the synthetic peptide (Figure 6). The isotopic distribution of the experimentally obtained spectrum for the intact, Cys179-modified peptide precisely matched the theoretical spectrum due to A2-IsoP modification (Figure 6A). Identification of the modified species with m/z 1115.578 was obtained by MS/MS, and comparison of the MS/MS spectrum of the modified and unmodified (m/z 1004.174) species shows the same peptide backbone (Figure 6B). When ozone was added, a notable increase in the quantity of cysteic acid (triply oxidized Cys179) was observed, with or without the presence of A2-IsoP (data not shown). Neither the singly or doubly-oxidized Cys179 peptide were observed in this experiment. The peptide was modified by A2-IsoP in the absence of ozone only to 10% of the +ozone level, and was not modified by F2-IsoP in the presence or absence of ozone (Figure 6C), suggesting this reaction is specific to the A2-IsoP maleamide in an ozone-catalyzed manner. The Cys residue was required because the mutant peptide Cys179Ala was not modified by ozone +/- A2-IsoP (data not shown). The proposed
structure corresponding to the A2-isoP modified IKK peptide as sequenced in this experiment, is shown in Figure 6D.
**Discussion**

**Absence of NQO1 regulates host responses to OZ by increasing production of A$_2$-isoprostane**

Epidemiologic studies have focused attention on several potential candidate gene polymorphisms that influence ozone-activated inflammation and airway hyperresponsiveness. In this report, we present evidence that the absence of one specific host factor, NQO1, that regulates reduction of cellular antioxidants, causes a shift in ozone-stimulated IsoP production resulting in increased production of precursors for the cyclopentenone IsoP, A$_2$-IsoP (Figure 7). Importantly, published reports demonstrate that there is a balance of F$_2$-IsoPs to E$_2$/D$_2$-IsoPs and A$_2$/J$_2$-IsoPs formed that is dependent on the relative reducing environment in the cell (32). The presence of NQO1 regenerates dihydroquinones such as $\alpha$-tocopherol which increase the intracellular reducing capacity. Following an oxidative stress, in the presence of sufficient levels of antioxidants, $\alpha$-tocopherol and reduced glutathione, there is shift towards formation of F$_2$-IsoPs. In contrast, when cellular antioxidants are depleted, following an oxidative stress, there is a shift towards formation of E$_2$/D$_2$-IsoPs and A$_2$/J$_2$-IsoPs. Reducing agents such as dithiothreitol, cysteine, $\beta$-mercaptoethanol, and glutathione supplementation increase F$_2$-isoP production supporting the importance of cellular redox status to determine the types of isoprostanes generated (24).

Previously, we demonstrated that in NQO1-null mouse lung, ozone exposure does not increase F$_2$-IsoP production (22). In this report, we show that ozone exposure of NQO1-null mice generated increased production of E$_2$/D$_2$-IsoPs, the precursors of A$_2$/J$_2$-IsoPs. This mechanism may be generalized to other diseases. For example, patients with Alzheimer’s Disease have increased levels of D$_2$- and E$_2$-isoprostane compared to F$_2$-isoprostane levels (23). D$_2$/E$_2$-IsoPs, and not F$_2$-IsoPs, are also increased in settings of traumatic brain injury in both animal models and in human CSF (33,34). Our report is one of the first to provide evidence for a molecular mechanism explaining an epidemiologic link between gene polymorphisms and host response to ozone. We speculate that other gene x environment interactions responsible for increased susceptibility to ozone may be due to a shift in cellular redox status affecting the generation of F$_2$- vs. A$_2$-IsoPs.

**NF-κB is a redox sensitive transcription factor**

Classically, NF-κB is a redox-sensitive transcription factor (35) and there is ample evidence that reactive oxygen species (ROS) including H$_2$O$_2$ and ionizing radiation activate NF-κB (36). Ozone exposure (7), and specifically a lipid ozonation product, hydroxynonane, activate both NF-κB and NF-IL6 to upregulate IL-8 expression in airway epithelial cells (10). NF-κB activation by LPS and cytokines requires ROS; and TNF$\alpha$ and IL-1β activate NADPH oxidases that contribute to ROS generation and are required for NF-κB activation (35). Ozone regulates TNF$\alpha$ expression in mice and this regulation is blocked in NF-κB p50-null mice (9).

However, depending on the cell type, the intracellular redox status and the kinetics of exposure, ROS may modify the sulfhydryl group in susceptible cysteine residues of key regulatory enzymes in the NF-κB activation pathway and inactivate NF-κB (35). The Cys179 residue in IKK is a critical target for reactive oxygen species that negatively regulate NF-κB activity. Acrolein, an $\alpha$, $\beta$-unsaturated aldehyde combustion product, blocks NF-κB activation and IL-8 expression in airway epithelial cells (37). Arsenite binds to and modifies the Cys-179 in the activation loop of IKKβ resulting in IKK inhibition and failure to activate NF-κB (38). Several other studies have also shown similar modification of Cys-179 in the IKKβ activation loop induced by Triterpenoid 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO) and the C-28 methyl ester (CDDO-Me) (39), Nimibolide (40), Piceatannol (41) and xanthohumol (42). Our report and others demonstrate that
cyclopentenone PGA₁ and A₂-IsoP both inhibit inflammation by direct inhibition of IKK activity (25,43), via oxidation of Cys-179 (43). The oxidant modifications of reactive cysteine residues may lead to either a reversible modification, such as a sulfenic group which may react with another cysteine to form a disulfide bond or may be reduced to return to a sulfhydryl group and permit enzyme reactivation. Sulfenics may also react with glutathione to form glutathionylation modifications that are also reversible. Importantly, our results demonstrate for the first time that in the presence of ozone, A₂-IsoP covalently binds to the sulfhydryl group of IKK Cys₁₇⁹.

**IsoPs have biological activity**

In this report, we demonstrate that A₂-IsoP inhibits ozone-induced inflammation. Furthermore, the increased presence of cyclopentenone precursors in NQO1-null mice lungs complement our previous report that NQO1-null mice have decreased ozone-induced F₂-IsoP production, IL-8 mRNA or KC protein expression, neutrophilic inflammation in the lung, and airway hyperresponsiveness (22). We also demonstrate for the first time that A₂-IsoP has biological activity in normal human airway epithelial cells. To date there has been limited data demonstrating direct biological activity of isoprostanes in murine models or human tissues.

Although F₂-IsoP in biological fluids is widely used as a quantitative index of *in vivo* oxidative stress, it has also been reported to have biological activity in the lung (44,45). 15-F₂-IsoP is a ligand for thromboxane receptors, FP receptors, and other yet uncharacterized receptors, resulting in airway smooth muscle contractility (46). It is not known whether F₂-IsoP stimulates primary human airway epithelial cell signaling or whether it can directly activate NF-κB and its downstream cascade.

A₂-IsoP is difficult to isolate and quantify in biological samples and fluids due to its reactivity with sulfhydryl groups, and this property has hampered investigations to determine its biological activities. In this report, we demonstrate that A₂-IsoP, at physiologic concentrations (nM) (47) and in concert with OZ, blocked NF-κB activation in the lung. A₂-IsoP modified a sulfhydryl moiety in Cys-179 within the active loop of IKK, resulting in inhibition of IKK activity. Inhibition of IKK results in failure to phosphorylate and degrade IκBα, impeding ozone-activation of NF-κB. These data are consistent with previous reports that A₂-IsoP inhibits NF-κB activation in LPS-activated macrophages and in human gestational tissues (25,26). Together, published reports and our results suggest the novel concept that A₂-IsoP may be generated under conditions of severe oxidative stress and may function as a “brake” to inhibit sustained inflammation. We propose that this molecular mechanism explains the epidemiologic observation that loss of NQO1 protects the host against ozone-induced lung inflammation.
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Figure legends

**Figure 1. Isoprostane quantitation in murine lungs.** C57BL/6 wild-type and NQO1-null mice were exposed to filtered air or ozone (OZ; 1 ppm, 3h) and then euthanized twenty four hours later for lung tissue collection. Mouse lungs were snap-frozen, stored at -80°C, and then extracted for E\textsubscript{2} & D\textsubscript{2}-isoprostane quantification by GC/NICI/MS. E\textsubscript{2} and D\textsubscript{2}-isoprostane quantitation is presented as ng/g lung tissue (mean ± SEM, n = 13-14 animals/group). * = wild-type OZ, significantly different from wild type-filter air (FA) control, p < 0.05; # = NQO1-null FA significantly different from wild-type FA control, p<0.05.

**Figure 2. Time course of ozone-induced IL-8 mRNA expression in NHBE cells in the presence or absence of A\textsubscript{2}-isoprostane.**

NHBE cells cultured at ALI were exposed to filtered air (FA) or ozone (OZ) over time (0.4 ppm, 0.5 - 5 h) and total RNA was isolated using Trizol reagent. IL-8 gene expression was determined using qRT-PCR, normalization to 18s rRNA and expression calculated by the \( \Delta\Delta CT \) method (A). Results were expressed as a percentage of the 30 minute filtered air control (mean ± SEM; n=10). * = OZ significantly different from corresponding FA control, p < 0.05. NHBE cells cultured at ALI were exposed to filtered air (control) or ozone (0.4 ppm, 2h [B and C] or 5 h [D and E]) in the presence or absence of A\textsubscript{2}-isoprostan (A2) (50 nM or 2.5 µM) for 1 h prior to and then during the exposure. Total RNA was isolated using Trizol. IL-8 gene expression was determined using qRT-PCR, normalization to 18s rRNA and expression compared to control treatment levels by the \( \Delta\Delta CT \) method (mean ± SEM; n=6-7). * = significantly different from control, p < 0.05. # = Ozone + A\textsubscript{2}-isoprostanes significantly different from Ozone alone, p < 0.05.
Figure 3. IL-8 protein concentrations in NHBE conditioned media following ozone +/- A$_2$-isop treatment. NHBE cells cultured at ALI were exposed to filtered air (FA) or ozone (OZ, 0.4 ppm, 5 h) in the presence or absence of A$_2$-isoP (2.5 µM) for a 60 min pretreatment and during OZ or FA exposure. Culture media was collected at the end of OZ exposure for ELISA quantitation of IL-8 (1:5 or 1:10 dilution). Two different NHBE donors were evaluated; (A) n= 9; (B) n=14; data are summarized as mean ±SEM; *, significantly different between OZ and FA, p<0.001; #, significantly different between OZ and OZ+ A$_2$-isoP, p< 0.05.
Figure 4. Time course of ozone-induced NF-κB activation in NHBE cells in the presence and absence of A₂-isoprostane.

NHBE cells cultured at ALI were exposed to filtered air (FA) or ozone (0.4 ppm, 30, 60 and 120 min; OZ) and then total cell lysates were collected. Cell lysate proteins (70 µg) were evaluated by Western blot analysis for phosphorylated-p65 (p-p65), total p65, and actin as a control for equal loading and transfer (A). The fold change between the time points was determined by densitometry and results were presented as a percentage of the corresponding FA control (B). NHBE cells cultured at ALI were exposed to filtered air (FA) or ozone (OZ; 0.4 ppm, for 30 min [C and D] or 60 min [E and F]) in the presence or absence of A₂-isoprostanes (A₂) (50 nM or 2.5 µM) for 1 h prior to and then during the exposure. At the end of exposure, total cell lysates (60-100 µg) were evaluated by Western blot analysis for p-p65, total p65 and actin. Results shown are representative of 4-6 independent experiments. The fold change between the time points was determined by densitometry and results were presented as a percentage of the corresponding FA control. Results were summarized graphically (mean ± SEM; n = 4-6). * = significantly different from filter air control, p < 0.05. # = Ozone + A₂-isoprostanes significantly different from Ozone alone, p < 0.05.

Figure 5: Time course of ozone-induced IKK activity in NHBE cells in the presence and absence of A₂-isoprostane

NHBE cultured at ALI were exposed to filtered air (FA) or ozone (0.4 ppm, 30, 60, or 120 min; OZ) and then total cell lysates (500 µg protein) were immunoprecipitated with specific antibodies to IKK enzymes. The resultant immunoprecipitates were assayed for IKK enzyme activity using GST IκBα as a substrate (A). Phosphorylation of IκBα detected by western analysis was used as a measure of IKK activity (upper panel). The amount of immunoprecipitated IKK enzyme was determined by reprobing the membranes with total anti-
IKKβ antibody (lower panel). Western results are representative of at least 2 independent time course experiments. NHBE cells cultured at ALI were exposed to filtered air (control) or ozone (OZ) (0.4 ppm, 30 min [B and C] or 60 min [D and E]) in the presence or absence of A2-isoprostanes (A2) (50 nM or 2.5 µM) for 1 h prior to and then during the exposure. At the end of exposure, total cell lysates (500 µg protein), were immunoprecipitated with specific antibodies to IKK enzyme, and the resultant immunoprecipitates were assayed for IKK enzymatic activity using GST-IkB-α as a substrate. Phosphorylation of IkB-α detected by Western blot analysis was used as a measure of IKK activity. The amount of immunoprecipitated IKK was determined by re-probing the membranes with total anti-IKKβ antibody. The fold change between the time points was determined by densitometry and results were presented as a percentage of the corresponding FA control. Results were summarized graphically from five separate experiments (mean ± SEM; n = 5-8). * = significantly different from control, p < 0.05. # = Ozone + A2-isoprostanes significantly different from Ozone alone, p < 0.05. Incubation with non-immune polyclonal rabbit IgG failed to immunoprecipitate IKK [A, lane 1, (IgG con)].

**Figure 6. Mass spectrometry analysis of IKK Cys-179 sulfoxide modification in the presence of Filtered Air or ozone and in the presence or absence of A2-isoprostane**

LC-MS identification of A2-IsoP derivatized C179 from synthetic IKKB peptide E172:K198.

**A)** Experimental and theoretical precursor ion isotope distribution of the E172:K198 peptide with an A2-IsoP modification ([M+3H]3+, 1115.5789 m/z). Mass accuracy error compared to theoretical was 0.01 ppm.

**B)** MS/MS spectra of A2-IsoP derivatized (top spectrum) and non-derivatized (bottom spectrum) E172:K198 peptide ([M+3H]+ precursor) localizing A2-IsoP modification to C179.

**C)** Extracted ion chromatograms of A2-IsoP modified E172:K198 peptide (1115.5789 ± 20 ppm) between +/- O3 and +/- A2-IsoP conditions indicated an O3-catalyzed mechanism of
derivatization. $O_3 + F_2$-isoP did not modify the peptide $(+O_3 + F_2)$.

**D** Theoretical structure of $A_2$-IsoP covalently modified IKKB $E_{172}^\#:K_{198}$ peptide.

**Figure 7.** *Hypothetical mechanism of ozone-induced and NQO1-mediated production of isoprostanes.* The presence or absence of NQO1 regulates the reduction-oxidation (redox) status of epithelial cells. In the presence of an oxidative stress such as ozone, isoprostanes are generated. The isoprostane generated is dependent on the redox status of the cells with $F_2$-isoprostane preferred under reducing conditions while $A_2$-isoprostane is preferred under oxidizing conditions. In the presence of ozone, $A_2$-isoprostane inhibits NF-κB activation resulting in blunted IL-8 upregulation and decreased neutrophilic inflammation.
FIGURE 1.
FIGURE 2.

A) Time course of relative IL-8 expression (% of 30 minute air control) with treatments: FA (O) and OZ (△).

B) Bar graph showing relative IL-8 mRNA expression with treatments: Control, A2 (50nM), Ozone (0.4ppm x 2h), and Ozone + A2.

C) Bar graph showing relative IL-8 mRNA expression with treatments: Control, A2 (2.5μM), Ozone (0.4ppm x 2h), and Ozone + A2.

D) Bar graph showing relative IL-8 mRNA expression with treatments: Control, A2 (50nM), Ozone (0.4ppm x 5h), and Ozone + A2.

E) Bar graph showing relative IL-8 mRNA expression with treatments: Control, A2 (2.5μM), Ozone (0.4ppm x 5h), and Ozone + A2.
FIGURE 4.

**A**

| Time (min) | FA | OZ |
|------------|----|----|
| 30         |    |    |
| 60         |    |    |
| 120        |    |    |

- p-p65(ser536)
- Actin
- p65

**B**

**C**

| A2-IsoP (μM) | FA | OZ |
|--------------|----|----|
| 0            |    |    |
| 0.05         |    |    |
| 2.5          |    |    |

- p-p65(ser536)
- Actin
- p65

**D**

**E**

| A2-IsoP (μM) | FA | OZ |
|--------------|----|----|
| 0            |    |    |
| 0.05         |    |    |
| 2.5          |    |    |

- p-p65(ser536)
- Actin
- p65

**F**
FIGURE 6.
Figure 7.
NADPH Quinone Oxidoreductase 1 Regulates Host Susceptibility to Ozone via Isoprostane Generation
Apparao B. Kummarapurugu, Bernard M. Fischer, Shuo Zheng, Ginger L. Milne, Andrew J. Ghio, Erin N. Potts-Kant, W. Michael Foster, Erik J. Soderblom, Laura G. Dubois, M. Arthur Moseley, J. Will Thompson and Judith A. Voynow

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