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Adrenal-derived stress hormones modulate ozone-induced lung injury and inflammation

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ABSTRACT

Ozone-induced systemic effects are modulated through activation of the neuro-hormonal stress response pathway. Adrenal demedullation (DEMED) or bilateral total adrenalectomy (ADREX) inhibits systemic and pulmonary effects of acute ozone exposure. To understand the influence of adrenal-derived stress hormones in mediating ozone-induced lung injury/inflammation, we assessed global gene expression (mRNA sequencing) and selected proteins in lung tissues from male Wistar-Kyoto rats that underwent DEMED, ADREX, or sham surgery (SHAM) prior to their exposure to air or ozone (1 ppm), 4 h/day for 1 or 2 days. Ozone exposure significantly changed the expression of over 2300 genes in lungs of SHAM rats, and these changes were markedly reduced in DEMED and ADREX rats. SHAM surgery but not DEMED or ADREX resulted in activation of multiple ozone-responsive pathways, including glucocorticoid, acute phase response, NRF2, and PI3K-AKT. Predicted targets from sequencing data showed a similarity between transcriptional changes induced by ozone and adrenergic and steroidal modulation of effects in SHAM but not ADREX rats. Ozone-induced increases in lung *Il6* in SHAM rats coincided with neutrophilic inflammation, but were diminished in DEMED and ADREX rats. Although ozone exposure in SHAM rats did not significantly alter mRNA expression of *Ifnγ* and *Il-4*, the IL-4 protein and ratio of IL-4 to IFNγ (IL-4/IFNγ) proteins increased suggesting a tendency for a Th2 response. This did not occur in ADREX and DEMED rats. We demonstrate that ozone-induced lung injury and neutrophilic inflammation require the presence of circulating epinephrine and corticosterone, which transcriptionally regulates signaling mechanisms involved in this response.

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1. Introduction

Ozone is a ubiquitous gaseous air pollutant and one of the major components of smog in urban areas (Cooper et al., 2014; Haagen-Smit, 1952). Due to its reactive nature, acute ozone inhalation causes oxidation of biomolecules such as proteins (Hemming et al., 2015; Kim et al., 2010) and lipids (Kadiiska et al., 2013; Thompson et al., 2013) in lung lining fluid and epithelial cells. An imbalance in the lung oxidant/antioxidant ratio has been widely reported after ozone exposure (Bromberg, 2016; Wiegman et al., 2014). Subsequent activation of pro-inflammatory signaling cascades involving mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase- protein kinase B (PI3K-AKT), and nuclear factor erythroid 2-related factor 2 (NRF2) (Yan et al., 2016) have been shown *in vitro* and *in vivo*, to increase transcription of cell adhesion molecules and pro-inflammatory cytokines (Bromberg and Koren, 1995; Chen et al., 2007; Montuschi et al., 2002). This activation results in the extravasation of innate immune cells, including neutrophils, to the lungs within hours after ozone exposure (Kim et al., 2011; Kirsten et al., 2013; Cabello et al., 2015; Williams et al., 2007; Auerbach and Hernandez, 2012). Ozone exposure has been shown to alter the balance between Th1 and Th2 phenotypes (Steerenberg et al., 1996). Th1-type cytokines are involved in immunity against intracellular pathogens where the effector cytokine IFNγ plays a pivotal role. Th2-type cytokines are involved in the immunity against extracellular parasites as well as development of type 1 hypersensitivity response seen in allergic asthma and are driven mainly by IL4 and IL13 (Berger, 2000). In mice, an ozone-induced Th2 phenotype shift was shown to depend on innate lymphoid cells (Kumagai et al., 2016).

Adverse ozone effects are not restricted to the lungs and multiple extra-pulmonary alterations have also been reported (Watkinson et al., 2001; Thomson et al., 2013). Ozone inhalation activates stress-responsive regions of the brain, including the nucleus tractus solitarius

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(NTS) where terminal fields of the lung vagal afferents overlap (Gackière et al., 2011). Ozone exposure also induces reflexively-mediated cardiovascular alterations, such as bradycardia and hypothermia, and decreases blood pressure (Akcılar et al., 2015; Uchiyama and Yokoyama, 1989; Gordon et al., 2014), indicating activation of the autonomic nervous system (Watkinson et al., 1996). It is postulated that nociceptive bronchial C-fibers stimulated by ozone inhalation transmit sensory information to the CNS and mediate systemic responses. These C-fibers modulate ozone-induced airway hyperresponsiveness but not hypothermia or bradycardia in rats (Jimba et al., 1995; Taylor-Clark and Undem, 2011).

Acute ozone inhalation induces systemic metabolic alterations including hyperglycemia, glucose intolerance, release of free fatty acids, activation of acute phase response, and muscle protein catabolism associated with a rise in circulating epinephrine and corticosterone levels (Bass et al., 2013; Miller et al., 2015, 2016a, 2016b). Further, performing an adrenal demedullation (DEMED) or adrenalectomy (ADREX) in rats, which diminishes the source of circulating catecholamines (synthesized in adrenal medulla upon sympathetic stimulation) and catecholamines plus steroid hormones (later synthesized in the adrenal cortex upon stimulation of HPA-axis), respectively, inhibits ozone-induced systemic metabolic impairment (Miller et al., 2016c). Importantly, pulmonary injury and inflammation induced by ozone exposure in rats that have undergone sham surgery (SHAM) are also diminished by ADREX (Miller et al., 2016c), highlighting a potential role of stress hormones in mediating ozone-induced pulmonary injury and inflammation. Although the mechanisms by which ozone induces local lung injury and inflammation are fairly well characterized, the role of circulating factors, such as stress hormones, in modulating pulmonary vascular leakage and extravasation of circulating immune cells has not been studied. We hypothesized that analysis of the expressed lung transcriptome in SHAM, DEMED, and ADREX rats exposed to ozone would elucidate potential mechanisms by which these hormones contribute to inflammation and injury in the lung following ozone exposure. Further, that decreased circulating levels of epinephrine and corticosterone in ADREX and DEMED rats would transcriptionally inhibit inflammatory modulators involved in ozone-induced neutrophilic inflammation while altering the balance of specific cytokine pools involved in immune function.

2. Materials and methods

2.1. Animals, surgeries and exposure

Lung tissue samples from healthy, male Wistar Kyoto (WKY) rats aged 12–13 weeks (Charles River Laboratories Inc., Raleigh, NC) were used from Miller et al., 2016c. SHAM, DEMED and ADREX surgeries were performed using aseptic sterile technique. Briefly, ketamine plus xylazine (50 mg plus 4 mg/kg body weight, i.p.) were used for anesthesia. Prior to surgery, rats received buprenorphine (0.02 mg/kg, subcutaneous) as an analgesic. Charles River Surgeons performed three types of sterile surgeries: control sham surgeries where all procedures were identical to the ADREX surgery except for the removal of adrenal gland (SHAM), bilateral adrenal demedullation where only the medulla portion of the adrenal glands was removed while the cortex was kept in place (DEMED), or bilateral total adrenalectomy where whole adrenal glands were removed (ADREX). Following 4 days of recovery, rats were exposed to either air or 1 ppm ozone, 4 h/day for 1 day or 2 consecutive days (1-D or 2-D) in whole body chambers under controlled flow, temperature and relative humidity. Ozone was generated by a silent arc discharge generator (OREC, Phoenix, Arizona). The chambers (Rochester style “Hinners”) flow was controlled by mass flow controllers (Coastal Instruments Inc., Burgaw, North Carolina) and the ozone concentrations were recorded using photometric ozone analyzers (API model 400, Teledyne Instruments; San Diego, California). Within 1 h of the final exposure, animals were euthanized *via* an overdose of pentobarbital (>200 mg/kg, i.p.). Bronchoalveolar lavage (BAL) was performed on the right lungs and left lung tissues were snap frozen in liquid nitrogen for storage at −80 °C for later analysis. Cell free BALF aliquots were stored at −80 °C for further analysis.

2.2. Lung RNA isolation, and quantification

A uniform portion of left lung (10–20 mg) was extracted for RNA isolation using reagents provided in RNeasy mini kits (Qiagen, Valencia, CA). RNA yield was determined using Qubit fluorometric quantitation (Thermo Fisher Scientific Inc., Waltham, MA).

2.3. RNA sequencing

mRNA was isolated from total RNA (500 ng each) using prepX polyA mRNA Isolation Kit (Wafergen Biosystems, Fremont, CA). Samples were run using manufacturer's protocol on the Apollo324 automated sample processing system for mRNA selection and continued on RNA-Seq library prep with Wafergen's PrepX mRNA 48 Protocol. The resulting cDNA libraries were PCR amplified for 15 cycles with indexing primers according to Wafergen's protocol. One microliter was taken from each library for quantitation by Qubit dsDNA HS Assay kit (Molecular Probes, Eugene, Oregon). The quality of libraries was checked by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the molar concentration of each library was estimated by using average molecular sizes from Bioanalyzer data and the concentration from Qubit measurement, and each library was diluted to 4 nM accordingly. The diluted libraries were again checked by Qubit to confirm the working concentrations and pooled to make the sample for sequencing run. The pooled libraries were denatured and diluted according to Illumina NextSeq 500 protocols (Illumina Inc., San Diego, CA). The final concentration for sequencing was 1.8 pM + 5% PhiX from Illumina. The sequencing data were stored in Illumina's BaseSpace-cloud.

2.4. Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

For qPCR, each RNA sample was diluted to 10 ng/μL and kept at −80 °C until the day of the experiment. One-step qPCR (SuperScript III, Invitrogen, Grand Island, NY) was run on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) using 50 ng of RNA. Primers containing a 6-carboxy-fluorescein (FAM dye) label at the 5′ end were purchased from Applied Biosystems (Foster City, CA) for the following genes: *β-actin* (Rn00667869_m1), tumor necrosis factor alpha (*Tnfα,* Rn99999017_m1), interleukin 6 (*IL-6,* Rn01410330_m1), interleukin 1 beta (*IL-1β,* Rn00580432_m1), interleukin 4 (*IL-4,* Rn01456866_m1), interferon gamma (*Ifnγ,* Rn00594078_m1), interleukin 5 (*IL-5,* Rn01459975_m1), and interleukin 13 (*IL-13,* Rn00587615_m1). Data were analyzed using ABI sequence detection software, version 2.2 using *β-actin* as an endogenous control. Expression of each sample was calculated as relative fold change over air-SHAM group at each time point (1-D or 2-D) using the $2^{-\Delta\Delta CT}$ method.

2.5. Cytokine protein quantification

BALF levels of cytokine proteins (IL-1β, IL-4, IL-5, IL-6, IL-10, IFN-γ, KC-GRO, TNF-α) were quantified using the V-PLEX proinflammatory panel 2 (rat) kit per manufacturer's protocol (Meso Scale Discovery, Gaithersburg, MD). The resulting electrochemiluminescence signals for each target protein in sample wells were detected using Meso Scale Discovery® electrochemiluminescence (MSD-ECL) platform (Mesoscale Discovery Inc., Rockville, MD). The values below the limit of detection were substituted with the lowest quantified value for each cytokine. Measurement of BALF inflammatory mediator proteins was restricted to 2-D only since ozone-induced inflammation peaks at this time point (Ward et al., 2015).