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Classical and Alternative Macrophage Activation in the Lung following Ozone-induced Oxidative Stress

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Abstract

Ozone is a pulmonary irritant known to cause oxidative stress, inflammation and tissue injury. Evidence suggests that macrophages play a role in the pathogenic response; however, their contribution depends on the mediators they encounter in the lung which dictate their function. In these studies we analyzed the effects of ozone-induced oxidative stress on the phenotype of alveolar macrophages (AM). Exposure of rats to ozone (2 ppm, 3 h) resulted in increased expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG), as well as heme oxygenase-1 (HO-1) in AM. Whereas 8-OHdG was maximum at 24 h, expression of HO-1 was biphasic increasing after 3 h and 48–72 h. Cleaved caspase-9 and beclin-1, markers of apoptosis and autophagy, were also induced in AM 24 h post-ozone. This was associated with increased bronchoalveolar lavage protein and cells, as well as matrix metalloproteinase (MMP)-2 and MMP-9, demonstrating alveolar epithelial injury. Ozone intoxication resulted in biphasic activation of the transcription factor, NFκB. This correlated with expression of monocyte chemotactic protein -1, inducible nitric oxide synthase and cyclooxygenase -2, markers of proinflammatory macrophages. Increases in arginase-1, Ym1 and galectin-3 positive anti-inflammatory/wound repair macrophages were also observed in the lung after ozone inhalation, beginning at 24 h (arginase-1, Ym1), and persisting for 72 h (galectin-3). This was associated with increased expression of pro-surfactant protein-C, a marker of Type II cell proliferation and activation, important steps in wound repair. These data suggest that both proinflammatory/cytotoxic and anti-inflammatory/wound repair macrophages are activated early in the response to ozone-induced oxidative stress and tissue injury.

Keywords

Ozone; oxidative stress; macrophage phenotype; heme oxygenase-1; autophagy; galectin-3

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Introduction

Ozone is a highly reactive oxidant that induces lung injury and impairs pulmonary mechanics (Uysal and Schapira, 2003). Toxicity is initiated by ozone-induced peroxidation of polyunsaturated fatty acids in membrane lipids and in lung lining fluid, resulting in the generation of reactive oxygen species, and a mixture of lipid ozonation products including lipoperoxyl radicals, hydroperoxides, malonydialdehyde, isoprostanes and alkenals such as 4-hydroxy-nonenal (Mustafa, 1990; Pryor *et al.*, 1996; Kafoury *et al.*, 1999; Rahman *et al.*, 2002). These reactive products cause oxidative stress in the lung. This leads to damage to the respiratory epithelium, disruption of alveolar epithelial barrier function, edema and inflammation (Hollingsworth *et al.*, 2007; Al-Hegelan *et al.*, 2011).

Evidence suggests that inflammatory macrophages accumulating in the lung in response to ozone-induced injury contribute to oxidative stress and pulmonary toxicity [reviewed in (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011)]. Thus, following exposure to products released from ozone-injured epithelial cells, lung macrophages are classically activated to release cytotoxic/proinflammatory mediators including reactive oxygen and nitrogen species, and tumor necrosis factor- α (TNF α) which promote tissue injury. This is supported by findings that blocking macrophages or cytotoxic mediators they release protects against ozone-induced lung injury (Pendino *et al.*, 1995; Cho *et al.*, 2001; Fakhrzadeh *et al.*, 2002; Toward and Broadley, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b). Accumulating data indicate that macrophages also play a protective role following ozone-induced lung injury, clearing oxidized products and cellular debris (Ishii *et al.*, 1998; Dahl *et al.*, 2007). They also augment lung antioxidant activity and release mediators that suppress inflammation and initiate wound repair (Reinhart *et al.*, 1999; Dahl *et al.*, 2007; Backus *et al.*, 2010). It appears that these activities are mediated by a distinct subpopulation of macrophages that is alternatively activated (Byers and Holtzman, 2011; Laskin *et al.*, 2011). The present studies demonstrate that oxidative stress caused by inhalation of ozone leads to activation of both cytotoxic/proinflammatory and anti-inflammatory/wound repair macrophages in the lung. Moreover, their appearance overlaps suggesting that processes of tissue injury and repair are initiated early in the pathogenic response to ozone.

Methods

Animals and exposure

Female specific pathogen-free Wistar rats (200–225 g) were obtained from Harlan Laboratories (IN). Animals were housed in filter top microisolation cages and maintained on food and water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Animals were exposed in groups of 4 to ultra-pure air (Messer Gas, Allentown, PA) or 2 ppm ozone for 3 h in a whole body Plexiglas chamber. Ozone was generated from oxygen gas via an ultraviolet-light ozone generator (Gilmont Instruments, Barrington, IL) and mixed with air. Concentrations inside the chamber were monitored using a Photometric ozone analyzer (model 400E, Teledyne Instruments, City of Industry, CA).

Sample collection

Animals were euthanized 3–72 h after exposure to air or ozone by intraperitoneal injection of Nembutal (250 mg/kg). The lung was perfused *in situ* via the portal vein with 50 ml of warm (37°C) perfusion medium (25 mM HEPES, 0.5 mM EGTA, 4.4 mM NaHCO₃ in HBSS, pH 7.3), followed by perfusion with Ca⁺²/Mg⁺²-free HBSS (22 mM HEPES, 4.2

mM NaHCO₃, pH 7.3) at a rate of 22 ml/min. Previous studies demonstrated that lung perfusion had no significant effect on the number of inflammatory cells in the lung (unpublished observations). For collection of bronchoalveolar lavage (BAL) fluid, the trachea was cannulated, and the lung lavaged by slowly instilling and withdrawing 10 ml cold PBS. BAL was centrifuged (300 x g, 10 min) and supernatants collected for measurement of protein content using a BCA Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin (BSA) as the standard. Lungs were excised and BAL cells recovered by lavage with an additional 40 ml (4 x 10 ml) of warm HBSS. Cells were combined with those recovered in the first 10 ml lavage, washed (300 x g, 10 min, 4°C) three times with HBSS containing 2% fetal bovine serum and then counted using a hemocytometer. Viability, as determined by trypan blue dye exclusion, was >98%. For cell differentials, slides were prepared using a Cytospin 4 (Shandon, Cheshire, England), and stained with Giemsa (Labchem Inc., Pittsburgh, PA). A total of 300 cells were counted by light microscopy.

Histology

Following lung perfusion, the left bronchus was clamped and the largest lobe of the lung inflated *in situ* via the trachea with PBS containing 3% paraformaldehyde. After 4 h on ice, the tissue was transferred to 50% ethanol. Histological sections (4 µm) were prepared and stained with hematoxylin and eosin. The extent of inflammatory changes including macrophage and neutrophil localization, alterations in alveolar epithelial barriers, and edema were assessed blindly by a board certified veterinary pathologist (Sherritta Ridgely, DVM). Images were also acquired at high resolution using an Olympus VS120 Virtual Microscopy System, scanned using VS-ASW version 2.4 software and viewed using OlyVIA version 2.4 software (Center Valley, PA). Lung sections from three rats per treatment group were evaluated.

Measurement of NFκB activity

Nuclear extracts were prepared using a kit from Active Motif (Carlsbad, CA). Five µg of nuclear protein samples were used in duplicate to quantify NFκB activity using the TransAM NFκB transcription factor assay kit (Active Motif).

Real time quantitative polymerase chain reaction

Total mRNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA). mRNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Standard curves were generated using serial dilutions of pooled cDNA samples. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT thermocycler. All PCR primers were generated using Primer Express 3.0 software (Applied Biosystems). Samples from 3 animals per treatment group were pooled for analysis and quantified relative to GAPDH mRNA expression. Forward and reverse primer sequences were Monocyte chemotactic protein (MCP)-1: CCA CTC ACC TGC TGC TAC TCA T, TCT CCA GCC GAC TCA TTG G; inducible nitric oxide synthase (iNOS): GGA TTT TCC CAG GCA ACC A, TCC ACA ACT CGC TCC AAG ATC; GAPDH: CCT GGA GAA ACC TGC CAA GTA T, CTC GGC CGC CTG CTT.

Immunohistochemistry

Tissue sections were deparaffinized. After antigen retrieval using citrate buffer (10.2 mM sodium citrate, 0.05% Tween 20, pH 6.0) and quenching of endogenous peroxidase with 3% H₂O₂ for 15 min, sections were incubated with goat serum (2–10%, room temperature, 1 h) to block nonspecific binding. This was followed by overnight incubation at 4°C with rabbit

polyclonal antibody to hemoxygenase (HO)-1 (1:200; Enzo Life Sciences, Plymouth Meeting, PA), caspase-9 (1:100; Cell Signaling (Danvers, MA), beclin-1 (1:200; Abcam, Cambridge, MA), matrix metalloproteinase (MMP)-2 (1:600; Abcam), iNOS (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA), cyclooxygenase (COX)-2 (1: 400; Abcam), Yml (1:200; Stem Cell Technologies, Vancouver, Canada), pro surfactant protein (SP)-C (1:500; Millipore, Billerica, MA), or mouse monoclonal antibody to 8-hydroxy-2'-deoxyguanosine (8-OHdG) (1:500; Abcam), arginase I (1:50; BD Biosciences, Rockville, MD), galectin-3 (1:400; R & D Systems, Minneapolis, MN), or appropriate IgG controls. Sections were then incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Binding was visualized using a Peroxidase Substrate Kit DAB (Vector Labs). Sections from three rats per treatment group were analyzed.

Western Blotting

BAL protein (2 µg) was fractionated on 10% SDS-PAGE or 4–12% gradient Tris-bis gels (Bio Rad, Hercules, CA) and transferred to polyvinylidene fluoride membranes (PVDF, 0.45 µm pore size; Millipore, Billerica, MA). Non-specific binding was blocked by incubation of the blots for 1 h at room temperature with 5% non-fat dry milk in 0.1% TBS-T buffer (0.02 M Tris-base, 0.137 M sodium chloride, 0.1 % Tween-20). Blots were incubated overnight at 4°C with rabbit polyclonal antibody to MMP-2 (1:2000, Abcam) or MMP-9 (1:1000, Abcam). After 4 washes in 0.1% TBS-T buffer, blots were incubated with anti-rabbit (1:20,000) HRP-conjugated secondary antibody (Cell Signaling, Danvers, MA) for 1 h at room temperature. Bands were visualized using an ECL or ECL+ detection system (GE, Healthcare Bio-Science Corp, Piscataway, NJ).

Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using student's t-test; a p value of 0.05 was considered statistically significant.

Results

Ozone is a potent pulmonary irritant known to cause oxidative stress (Bocci, 2006; Ciencewicz *et al.*, 2008; Yang and Omaye, 2009). Consistent with this activity, we observed a time-related increase in the DNA oxidation product, 8-OHdG in alveolar macrophages, as well as epithelial cells, which was most notable 24 h after ozone exposure (Figure 1). Subsequently 8-OHdG levels declined. The antioxidant HO -1 also increased in alveolar macrophages following ozone exposure, however this response was biphasic, occurring initially at 3 h and then again at 48–72 h (Figure 1). Oxidative stress has been linked to necrotic cell death, as well as to apoptosis and autophagy (Kirichenko *et al.*, 1996; Kosmider *et al.*, 2010; Li *et al.*, 2010; Ryter and Choi, 2010; Jin *et al.*, 2012). Following ozone inhalation, a rapid (within 3 h) and persistent (up to 48 h) increase in expression of cleaved caspase-9 was observed in alveolar macrophages (Figure 1), suggesting initiation of stress-induced mitochondrial apoptosis (Hu *et al.*, 2006). A persistent increase in expression of beclin-1, a marker of autophagy (Cao and Klionsky, 2007; Kang *et al.*, 2011), was also noted. However, this response was delayed, relative to cleaved caspase-9, and not evident until 24 h post-exposure. Alveolar epithelial cell cytotoxicity was also observed after ozone intoxication, as reflected by increased protein and inflammatory cell accumulation in BAL (Figure 2). Whereas protein levels increased within 3 h and remained elevated for 48 h, cell number increased more gradually, reaching maximum levels after 24–72 h. Differential analysis revealed that the majority (>97%) of BAL cells were macrophages at all post-exposure times.

Histologic sections were next examined to further assess ozone-induced structural and inflammatory changes in the lung. Ozone intoxication resulted in neutrophilic infiltration into the lung in peribronchiolar regions, along with patchy, mild thickening of alveolar septa and increased numbers of macrophages in the tissue (Figure 3). These effects were time-related becoming most prominent 48 h after exposure.

Matrix metalloproteinases including MMP-2 and MMP-9 have been reported to disrupt tight junctions, a process thought to be important in ozone-induced alveolar epithelial damage and lung hyperpermeability (Kenyon *et al.*, 2002; Feng *et al.*, 2011; Triantaphyllopoulos *et al.*, 2011). After ozone exposure, levels of MMP-2 and MMP-9 were increased in BAL at 3–24 h, and 3–12 h, respectively (Figure 4). MMP-2 expression was also observed in histologic sections in alveolar macrophages which was most notable 24 h after ozone inhalation (Figure 4).

We next analyzed the consequence of ozone-induced oxidative stress in alveolar macrophages. NF- κ B is a redox sensitive transcription factor that plays a key role in regulating macrophage production of cytotoxic and proinflammatory mediators (Hanada and Yoshimura, 2002). We found that NF- κ B p65 activity increased rapidly in alveolar macrophages following ozone exposure (Figure 5). This was evident within 3 h and was followed by a decrease at 6 h, and a secondary increase at 12 h which persisted for 48 h. In contrast, no significant changes were observed in alveolar macrophage NF- κ B p50 nuclear binding activity.

Activation of NF- κ B in macrophages culminates in the production of proinflammatory/cytotoxic proteins including MCP-1, iNOS and COX-2, each of which has been implicated in tissue injury (Zhao *et al.*, 1998; Fakhrzadeh *et al.*, 2002; Sung *et al.*, 2002; Tsatsanis *et al.*, 2006; Murugan and Peck, 2009). A marked increase in MCP-1 mRNA expression was detected in alveolar macrophages after ozone inhalation; this response was biphasic, initially peaking at 12 h and then at 72 h (Figure 6). A biphasic increase in iNOS mRNA was also evident at 6 h and 48 h post ozone exposure. This was associated with increased iNOS protein expression in alveolar macrophages, which was most notable 24 h after ozone (Figure 7). Within 3 h of ozone exposure, increased expression of COX-2 protein was also observed in alveolar macrophages; this persisted for 48 h before returning to control levels (Figure 7).

Evidence suggests that alternatively activated macrophage subpopulations play a role in suppressing inflammation and initiating wound repair after acute injury (Byers and Holtzman, 2011). Arginase-1, Ym-1 and galectin-3 are markers of alternatively activated macrophages (MacKinnon *et al.*, 2008; Nair *et al.*, 2009; Feola *et al.*, 2010; Gibbons *et al.*, 2011). A time-related increase in expression of each of these proteins was observed in alveolar macrophages following ozone inhalation (Figure 8). Interestingly, this was evident within 3 h of exposure. Whereas arginase-1 and Ym-1 positive macrophages remained prominently in the tissue for 24 h before declining, galectin-3 positive cells persisted for at least 72 h after ozone exposure; galectin-3 positive macrophages were also significantly enlarged and exhibited a foamy appearance. These markers of alternatively activated macrophages were not detectable in lung sections from control animals. The presence of alternatively activated alveolar macrophages was correlated with a rapid (within 3 h) and persistent increase in pro-SP-C expression, an indicator of Type II cell proliferation and activation, important steps in tissue repair (Figure 9).

Discussion

Ozone is a highly reactive molecule that directly or indirectly through the generation of free radicals, oxidizes proteins, lipids and DNA. The resulting oxidation products, together with reactive species produced by inflammatory cells, play a key role in ozone-induced tissue injury (Mustafa, 1990; Pryor *et al.*, 1996; Kafoury *et al.*, 1999; Rahman *et al.*, 2002). The present studies demonstrate that lung macrophages are highly sensitive to ozone-induced oxidative stress, developing into subpopulations displaying classically and alternatively activated phenotypes. Evidence suggests that these macrophage subpopulations play distinct roles in the inflammatory response to tissue injury. Thus, while classically activated macrophages promote tissue injury, alternatively activated macrophages participate in tissue repair [reviewed in (Laskin *et al.*, 2011)]. The fact that both of these cell populations appear in the lung within 24 h of ozone exposure suggests that processes of injury and repair occur rapidly in response to oxidative stress.

8-OHdG is a major DNA oxidation product. It has been identified in nasal respiratory epithelium and in urine from humans exposed to urban air pollution containing ozone, as well as in the urine of mice exposed to inhaled ozone, and it has been proposed as a biomarker of oxidative DNA damage induced by ambient air pollution (Calderon-Garciduenas *et al.*, 1999; Feng *et al.*, 2001; Chuang *et al.*, 2007; Ren *et al.*, 2011). Following ozone inhalation, we detected 8-OHdG in the nucleus of alveolar macrophages, suggesting that these cells are a target for oxidative DNA damage. This most likely results from the combined actions of ozone and its oxidation products, and reactive oxygen species generated by inflammatory leukocytes following ozone inhalation. This is supported by findings that increases in 8-OHdG in the lungs of mice exposed to diesel exhaust particles are directly correlated with alveolar macrophage production of hydroxyl radicals (Tokiwa *et al.*, 1999; Li *et al.*, 2008).

Oxidative stress is associated with both non-programmed (cytotoxicity) and programmed (apoptosis) cell death, as well as autophagy (Kirichenko *et al.*, 1996; Scherz-Shouval and Elazar, 2007; Kosmider *et al.*, 2010; Li *et al.*, 2010; Ryter and Choi, 2010; Jin *et al.*, 2012). We found evidence of each of these processes in the lung following ozone intoxication. Thus, increases in BAL protein were noted within 3 h of ozone inhalation, demonstrating alveolar epithelial necrosis/cytotoxicity. This was associated with a persistent increase in BAL inflammatory macrophages, a characteristic response to necrotic cell death. Ozone-induced oxidative DNA damage in alveolar macrophages was also associated with early markers of apoptosis (cleaved caspase-9), and autophagy (beclin-1). Expression of both cleaved caspase-9 and beclin-1 in alveolar macrophages reached a maximum 24 h post-exposure, a time coordinate with peak classical activation of these cells. Autophagy is a dynamic process controlling the turnover of cellular organelles and proteins, which is thought to be essential for the maintenance of cellular homeostasis, and for protection against oxidative stress (Jin *et al.*, 2012). Findings of induction of autophagy in alveolar macrophages following ozone intoxication are novel, and may represent a mechanism for limiting the proinflammatory/cytotoxic activity of these cells, and promoting the resolution of inflammation. This is supported by recent studies demonstrating that autophagy functions to balance the detrimental and beneficial effects of inflammation [reviewed (Levine *et al.*, 2011)].

MMP-2 and MMP-9 are proteases that degrade extracellular matrix proteins and disrupt tight junctions; they have been implicated in the pathogenesis of acute lung injury, asthma, fibrosis and chronic obstructive pulmonary disease (Srivastava *et al.*, 2007; Oikonomidi *et al.*, 2009). MMPs have been reported to be released by macrophages in response to oxidative stress (Yoshida *et al.*, 2001; Yoshida and Whitsett, 2006). We found that ozone exposure

resulted in increased levels of MMP-2 and MMP-9 in BAL, and increased MMP-2 expression in alveolar macrophages. These findings are in accord with previous reports of increased MMP-2 and MMP-9 in lungs of animals exposed to ozone and/or particulate matter (Kenyon *et al.*, 2002; Thomson *et al.*, 2005). The observation that MMP-2 persisted in macrophages and in BAL up to 48 h after ozone, are consistent with a role for this protease in inducing alveolar epithelial damage (Hayashi *et al.*, 1996; Bakowska and Adamson, 1998; Gushima *et al.*, 2001). Recent studies suggest that MMP's contribute to signaling pathways leading to both apoptosis and autophagy in breast cancer cells (Augustin *et al.*, 2009). It remains to be determined if they play a role in these signaling pathways in alveolar macrophages.

NF κ B is a redox sensitive transcription factor that regulates the activity of proinflammatory mediators important in host defense and tissue injury. It is also considered a marker of classically activated macrophages (Wilson *et al.*, 2005; Lawrence, 2011). In response to ozone-induced oxidative stress, NF κ B p65 nuclear binding activity rapidly (within 3 h) and transiently increased in alveolar macrophages; this was followed by a decline and then a secondary increase which peaked at 24 h. This biphasic increase most likely reflects early classical activation of resident alveolar macrophages and subsequent activation of infiltrating inflammatory macrophages. Our findings of biphasic increases in iNOS and MCP-1 mRNA, proinflammatory proteins known to be expressed by classically activated macrophages (Zhang *et al.*, 2007; Hussain *et al.*, 2011), are consistent with this idea. Another marker of classical macrophage activation, COX-2, was also upregulated in alveolar macrophages after ozone exposure. The fact that COX-2 expression peaked 24 h post exposure, a time when BAL cell number was increased, suggests that it is infiltrating inflammatory cells that are the major cell type undergoing phenotypic polarization into classically activated macrophages. This is supported by previous findings that macrophages accumulating in the lung in response to ozone produce tumor necrosis factor- α and interleukin-1, and generate increased quantities of reactive oxygen and nitrogen species (Pendino *et al.*, 1994; Ishii *et al.*, 1997; Fakhrzadeh *et al.*, 2002, 2004b). The observation that inhibiting these cells with gadolinium chloride, or blocking proinflammatory mediator activity or NF κ B, protects against ozone toxicity provide support for a role of classically activated macrophages in the early pathogenic response (Pendino *et al.*, 1995; Cho *et al.*, 2001; Fakhrzadeh *et al.*, 2002; Toward and Broadley, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b).

Alternatively activated macrophages are known to play a role in suppressing inflammation and inducing wound repair (Byers and Holtzman, 2011; Laskin *et al.*, 2011). Following ozone exposure, we observed an accumulation of alternatively activated macrophages in the lung, as determined by their expression of arginase-1, Ym1 and galectin-3. Whereas the appearance of arginase-1 and Ym1 positive macrophages was transient reaching a maximum 24 h after ozone exposure, galectin-3 expressing macrophages persisted in the lung for at least 72 h. Findings that the kinetics of appearance of these markers is different suggest that there are multiple subpopulations of alternatively activated macrophages responding to ozone that make distinct contributions to tissue repair. Thus, while Ym1 and arginase-1 positive macrophages may act to suppress inflammation, galectin-3 positive macrophages may contribute more to tissue repair (Kasper and Hughes, 1996; Pesce *et al.*, 2009; Sundblad *et al.*, 2011). The fact that galectin-3 positive cells are morphologically distinct from Ym1 and arginase-1 positive macrophages and enlarged suggests that they are activated and supports the idea of macrophage subpopulation heterogeneity. Galectin-3 has been reported to promote classical activation of macrophages (Dragomir *et al.*, 2012). Differences between our findings may be due to the tissue specific actions of galectin-3.

Of interest was our observation that peak accumulation of both classically activated macrophages and subpopulations of alternatively activated macrophages occurred 24 h post ozone exposure. These results suggest that inflammatory processes related to both tissue injury and repair are triggered relatively early after ozone intoxication. Following alveolar epithelial injury, Type II cells proliferate to repair damaged epithelium (Chang *et al.*, 1992). SP-C is a pulmonary surfactant synthesized by Type II cells and a marker for activation and proliferation these cells (Beers *et al.*, 1992). Our findings that pro-SPC is rapidly (within 3 h) upregulated in Type II cells following ozone exposure and that this response persists, provides additional support for the idea that tissue repair is initiated early after ozone intoxication.

Induction of HO-1 is a cellular response to oxidative stress (Ryter and Choi, 2005; Raval and Lee, 2010; Wu *et al.*, 2012). Following ozone inhalation, an increase in HO-1 was noted in alveolar macrophages which is consistent with our findings of ozone-induced DNA oxidation in these cells. Interestingly, induction of HO-1 was biphasic, occurring at 3 h and at 48–72 h. This may reflect distinct functions of HO-1 in alveolar macrophages. Thus, while early in the pathogenic response to ozone, macrophages upregulate HO-1 to mitigate oxidative stress and DNA damage (Takahashi *et al.*, 1997; Hisada *et al.*, 2000), at later times it may be involved in suppressing classically activated proinflammatory macrophages and promoting their transition to an anti-inflammatory/wound repair phenotype (Otterbein *et al.*, 2000; Devey *et al.*, 2009; Immenschuh *et al.*, 2010). This is thought to be due to HO-1 induced suppression of NF- κ B activation and proinflammatory activity (Devey *et al.*, 2009). Our observation that increased HO-1 expression in alveolar macrophages 48–72 h post ozone exposure is correlated with decreased NF- κ B activity and increasing expression of galectin-3 in lung macrophages are in accord with this late anti-inflammatory role of HO-1 in pulmonary toxicity.

The present studies demonstrate that lung macrophages are highly sensitive to ozone-induced oxidative stress. Moreover, this may contribute to the regulation of alveolar macrophage phenotype and function. These findings are novel and indicate that modulating the activity of macrophages by altering the balance of oxidants and antioxidants may be an effective approach to mitigating lung injury induced by ozone.

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Research Highlights

- Alveolar macrophages contribute to oxidative stress and pulmonary toxicity induced by ozone inhalation
- Both proinflammatory/cytotoxic and antiinflammatory/wound repair macrophages are activated early in the pathogenic response to ozone-induced oxidative stress and tissue injury
- Oxidative stress may contribute to the regulation of macrophage phenotype and function

Figure 1.

Effects of ozone on markers of oxidative stress, apoptosis and autophagy. Lung sections, prepared 3–72 h after exposure of rats to air or ozone, were stained with antibody to 8-OHdG, HO-1, cleaved caspase-9 or beclin-1. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 rats per treatment group is shown (Original magnification, x1000).

Figure 2.

Effects of ozone on BAL protein content and cell number. BAL was collected 3–72 h after exposure of rats to air or ozone. *Upper panel:* Cell-free supernatants were analyzed in triplicate for protein content using a BCA protein assay kit. *Lower panel:* Viable cells were enumerated by trypan blue due exclusion. Each bar is the average $\pm$ SE (n = 9–11 rats).

*Significantly different (p < 0.05) air-exposed animals.

Figure 3.

Effects of ozone on lung histology. Lungs sections, prepared 3–72 h after exposure of rats to air or ozone, were stained with H & E. Images were acquired using a VS120 Virtual Microscopy system. One representative lung section from 3 rats per treatment group is shown. *Right panels:* Original magnification, x400; *Left panels:* Original magnification, x48; circles indicate regions of high power magnification in right panels.

Figure 4. Effects of ozone on MMP-2 and MMP-9. BAL and lung tissue were collected 3–72 h after exposure of rats to air or ozone. *Upper Panel:* BAL was analyzed for MMP-2 and MMP-9 by Western blotting. One representative gel from 3 separate experiments is shown. *Lower panel:* Sections were stained with anti-MMP-2 antibody or IgG control. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 rats per treatment group is shown (Original magnification, x1000).

Figure 5.

Effects of ozone on NFκB nuclear binding activity. Alveolar macrophages were collected 3–72 h after exposure of rats to air or ozone. Nuclear extracts were analyzed in duplicate for p65 and p50 NFκB activity using a TransAM NFκB kit. Data are presented as fold-change relative to air control animals. Each bar is the average $\pm$ SE (n = 3 rats). *Significantly different (p < 0.05) from air-exposed animals.

Figure 6.

Effects of ozone on inflammatory mediator gene expression. Alveolar macrophages were collected 3–72 h after exposure of rats to air or ozone. mRNA was extracted, pooled, and analyzed in triplicate for MCP-1 and iNOS gene expression. Data were normalized to GAPDH and presented as fold-change relative to air control. Each bar is the average $\pm$ SE (n = 3 rats). *Significantly different (p < 0.05) from air-exposed animals.

Figure 7.

Effects of ozone on iNOS and COX-2 protein expression. Lung sections, prepared 3–72 h after exposure of rats to air or ozone, were stained with antibody to iNOS or COX-2.

Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 rats per treatment group is shown (Original magnification, x1000).

Figure 8.

Effects of ozone on arginase 1, Ym1 and galectin 3 protein expression. Lung sections, prepared 3–72 h after exposure of rats to air or ozone, were stained with antibody to arginase 1, Ym1 or galectin 3. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 rats per treatment group is shown (Original magnification, x1000).

Figure 9.

Effects of ozone on pro-SP-C protein expression. Lung sections, prepared 3–72 h after exposure of rats to air or ozone, were stained with antibody to pro-SP-C. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 rats per treatment is shown (Original magnification, x1000).