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Modeling Vascular Inflammation and Atherogenicity after Inhalation of Ambient Levels of Ozone: Exploratory Lessons from Transcriptomics

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Abstract

Background: Epidemiologic studies have linked inhalation of air pollutants such as ozone to cardiovascular mortality. Human exposure studies have shown that inhalation of ambient levels of ozone causes airway and systemic inflammation and an imbalance in sympathetic/parasympathetic tone.

Methods: To explore molecular mechanisms through which ozone inhalation contributes to cardiovascular mortality, we compared transcriptomics data previously obtained from bronchoalveolar lavage (BAL) cells obtained from healthy subjects after inhalational exposure to ozone (200ppb for 4h) to those of various cell samples from 11 published studies of patients with atherosclerotic disease using the Nextbio genomic data platform. Overlapping gene ontologies that may be involved in the transition from pulmonary to systemic vascular inflammation after ozone inhalation were explored. Local and systemic enzymatic activity of an overlapping up-regulated gene, matrix metalloproteinase-9 (MMP-9), was measured by zymography after ozone exposure.

Results: A set of differentially expressed genes involved in response to stimulus, stress, and wounding were in common between the ozone and most of the atherosclerosis studies. Many of

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Declaration of interest statement

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these genes contribute to biological processes such as cholesterol metabolism dysfunction, increased monocyte adherence, endothelial cell lesions, and matrix remodeling, and to diseases such as heart failure, ischemia, and atherosclerotic occlusive disease. Inhalation of ozone increased MMP-9 enzymatic activity in both BAL fluid and serum.

Conclusions: Comparison of transcriptomics between BAL cells after ozone exposure and various cell types from patients with atherosclerotic disease reveals commonly regulated processes and potential mechanisms by which ozone inhalation may contribute to progression of pre-existent atherosclerotic lesions.

Keywords

Vascular inflammation; Atherosclerosis; Systemic inflammation; Cardiovascular disease; Air pollution; Ozone; Airway inflammation; Gene expression; Transcriptomics; Leukocyte trafficking; Matrix Metalloproteinase-9

INTRODUCTION

Atherosclerotic vascular disease, a condition affecting over 15.4 million Americans, is a chronic inflammatory disease in which fatty deposits, inflammatory cells, and scar tissue accumulate within artery walls (Go et al. 2014, Libby et al. 2011, Pant et al. 2014). To reduce the health impact of cardiovascular disease (CVD), it is necessary to understand the possible environmental factors that contribute to the underlying pathology of cardiovascular disease, namely, the atherosclerotic plaque and subsequently plaque rupture. Ozone is a major constituent of air pollution to which millions of people are regularly exposed. When inhaled, ozone interacts with airway lining fluid to produce reactive oxygen species, resulting in oxidative injury (Balmes et al. 1997, Castleman et al. 1980, Chen et al. 2007, Chen et al. 2006, Koren et al. 1989, Kumarathasan et al. 2015, Leikauf et al. 1995, Leroy et al. 2015, Michaudel et al. 2016, Pino et al. 1992). Many epidemiological studies have provided substantial evidence that exposure to air pollution and its ozone component may contribute to increased respiratory and cardiovascular mortality (Bell et al. 2004, Brook et al. 2010, Jerrett et al. 2009, Ruidavets et al. 2005, Suissa et al. 2013, Turner et al. 2016). Although the biology of respiratory health effects of exposure to ozone have been extensively studied, the underlying molecular mechanisms by which inhalation of ozone may contribute to cardiovascular mortality are largely unknown.

Two controlled human exposure studies have investigated possible mechanisms by which ozone inhalation may contribute to CVD. These studies have documented systemic inflammation and autonomic imbalance with short-term inhalation of ambient levels of ozone in young healthy volunteers (Arjomandi et al. 2015, Devlin et al. 2012). A larger human exposure study in healthy older adults was recently concluded but its results are as of yet unpublished (ClinicalTrial.gov identifier NCT01487005). Several exposure studies have shown that inhalation of ambient levels of ozone causes local and systemic inflammation with recruitment of granulocytes and macrophages into the lung (Arjomandi et al. 2005, Arjomandi, et al. 2015, Chuang et al. 2009, Hiraiwa and van Eeden 2013). It is well established that systemic inflammation is a mediator in the pathogenesis of CVD (Chuang, et al. 2009, Glurich et al. 2002, Suwa et al. 2002, Taleb 2016, Willerson 2002, Willerson and

Ridker 2004), and macrophages are thought to be a major culprit in the development of atherosclerotic plaques (Dickhout et al. 2008, Gui et al. 2012, Liberale et al. 2016, Moore et al. 2013, Moore and Tabas 2011). Thus, a potential mechanism by which ozone exerts its cardiovascular effects may be through induction of local airway inflammatory processes that could then expand systemically (Bell, et al. 2004, Brook, et al. 2010, Jerrett, et al. 2009, Ruidavets, et al. 2005, Suissa, et al. 2013).

The goal of this study was to identify the relevant ozone-induced biological processes involved in eliciting and/or exacerbating a systemic inflammatory state that may contribute to atherosclerosis. We hypothesized that examination of differentially regulated processes due to ozone exposure identifies pathways that may be involved in pollutant-induced development and/or progression of atherosclerosis and ultimately cardiovascular mortality. To do this, we analyzed the gene expression of airway inflammatory cells from bronchoalveolar lavage (BAL) of healthy adults after exposure to clean air and high ambient levels of ozone (200 parts per billion, ppb), and compared that data with those of samples from patients with atherosclerotic disease from 11 studies using an *in silico* approach. Comparative gene expression analysis revealed the presence of a common subset of inflammatory chemokine, cytokine, and matrix remodeling genes, which may play important roles in the progression and/or predisposition of existing atherosclerotic plaques for arterial thrombotic events. As a proof-of-concept to provide support for the assumption that local airway inflammation could propagate systemically, we also examined the enzymatic activity of soluble matrix metalloproteinase (MMP)-9, the gene for which was differentially expressed in both CVD and ozone studies, in archived BAL and blood samples obtained from subjects in the current ozone exposure study.

METHODS

Study Overview.

The ozone exposure study design and the details of subjects, exposures, and bronchoscopy have been previously described in detail (Arjomandi, et al. 2015, Leroy, et al. 2015). Briefly, 19 healthy volunteers were exposed to clean filtered air (0 ppb), and medium (100 ppb), and high (200 ppb) ambient levels of ozone in a crossover counter-balanced fashion for 4 hours in a climate-controlled chamber followed by bronchoscopy with BAL 20 hours later. A minimum of 3 weeks was allotted between exposures to allow for recovery from any inflammation or injury sustained from the prior session. RNA was extracted from BAL cell pellet samples and was processed using RLT buffer (Qiagen) and RNeasy kit (Qiagen) as previously described (Leroy, et al. 2015). Gene expression data were obtained using Affymetrix platform (GeneChip® HumanGene 1.0 ST array) and analyzed using the Affymetrix GeneChip® Scanner 3000 7G and Affymetrix GeneChip® Command Console® Software (NCBI Gene Expression Omnibus Accession Number GSE58682) as previously described (Leroy, et al. 2015). To amplify the number of identified differentially expressed genes (DEGs) in this exploratory study, only data for 0 and 200ppb exposures were used in the analysis.

Subsequently, cardiovascular disease (CVD) studies with gene expression data (biosets) were identified using NextBio gene analysis application (Illumina; CA, USA), and using an

in silico approach, the DEGs of the BAL cell pellet were compared to DEGs from those biosets. Cell types and tissue samples from each cardiovascular study can be found in Table 1. Genes were filtered using a p-value cutoff of 0.05, with no multiple-testing correction. To ascertain that a reasonable number of commonly DEGs have been identified for this exploratory meta-analysis between transcriptomics data from CVD and ozone exposure studies, unadjusted p-values with no correction for multiple testing were used as per common practice in transcriptomics correlational studies using NextBio platform. The resulting set of genes was further filtered using a fold-change difference cutoff of ≥1.2 between the average intensity in test and control groups. Only genes with valid ENTREZ gene identification symbols were included in further analyses. Next, analyses of common Gene Ontology (GO) terms were queried in comparisons between the ozone and each CVD study to provide a ranked output list of GO terms that were highly overlapped in NextBio and Ingenuity Pathway Analysis (IPA; QIAGEN Inc., California, USA).

NextBio Search Strategy and Selection Criteria.

To identify studies relevant to atherosclerosis and coronary artery disease, we searched the NextBio electronic database, limiting the search to studies with publicly uploaded gene expression data (NextBio, www.nextbio.com; Cupertino, CA). The keywords used for the search were “myocardial infarction,” “atherosclerosis of artery,” “cardiovascular disease,” “atherosclerotic occlusive disease,” “myocardial disease,” “heart disease,” “stricture of artery,” and “coronary artery disease.” The Nextbio search covered studies present in the database to August 1st, 2016. Studies were filtered to only include those with RNA expression data on Homo Sapiens, and to exclude populations that did not match that of our study (e.g., Pediatric patients).

NextBio Data Processing.

For Affymetrix data, CEL files were processed using RMA normalization, and the summarized data were analyzed. When CEL files were unavailable, the existing summarized data are imported. As part of the NextBio analysis protocol (Kupershmidt et al. 2010) data were initially examined using diagnostic plots, such as box plots, for each study. To examine data quality and experimental design assumptions further, hierarchical clustering of samples was used to assess separation of samples visually, according to the treatment or test factors. In addition, an analysis of variance (ANOVA) F test was performed to compare the various factors (ie. treatment or control groups) in each study.

Nextbio Data Filtering and Differential Expression Statistical Analyses.

For independent sample comparison, a two-sample t-test was performed on each gene, comparing the treated group to the control group (Kupershmidt, et al. 2010). For paired samples, a paired t-test was performed. Genes were filtered using a p-value cutoff of 0.05, with no multiple-testing correction. The resulting set of genes was further filtered using a fold-change difference cutoff of ≥1.2 between the average intensity in test and control groups. To compare biosets between experiments, fold change was used as the default ranking, as it has been shown to give better concordance across platforms than p-values from statistical tests. Differentially expressed genes (DEGs) that were similarly up- or down-regulated in both the ozone study and the compared cardiovascular study (CVD) studies

were described as having a “concordant” correlation; DEGs that were in common but were regulated in opposite directions between the ozone study and the compared CVD studies were described as having a “discordant” correlation.

Gene Expression Data Processing in NextBio.

Data processing in NextBio is described at www.nextbio.com (Kupershmidt, et al. 2010). Briefly, CEL files were processed using Robust Multi-Array Average normalization, examined using diagnostic plots and hierarchical clustering, and then summarized data were analyzed using analysis of variance F test (ANOVA) to compare the treatment versus control groups for each study analyzed. Fold changes were used as the default ranking, as it gives better concordance across platforms than p-values from statistical tests(Shi et al. 2005).

NextBio Platform Meta-Analysis and Disease Atlas.

Each CVD study was compared independently to the ozone study using the NextBio meta-analysis tool to determine genes, GO terms, and diseases commonly associated between each bioset. Significant genes and GO terms (p-value<0.05) were then compared among all CVD and ozone meta-comparisons to identify relevant processes. To investigate the role of the identified subgroup of genes that were highly differentially regulated in all 11 CVD studies as well as ozone, we searched for diseases, traits, and conditions associated with each of the genes.

Ingenuity Pathway Analysis (IPA) Comparison Study.

The IPA software, which provides common canonical, disease, and upstream pathways between the selected studies, was used to run comparison analyses of the DEGs from the ozone and 11 CVD studies (Ingenuity Systems, www.ingenuity.com; Redwood City, CA). Biosets of ozone and 11 CVD studies were downloaded from the Nextbio database. The top (based on p-value) 300 DEGs from these biosets (or total number of genes if <300 were present in an individual bioset) were selected for analysis in the IPA Knowledge Base. All selected genes displayed a fold-change ≥1.2 and p-value <0.05.

Measurement of MMP-9 and MMP-2 Gelatinase Activity in BAL and Serum.

The gelatinase activity of the proteins of two of the DEGs, MMP-9 and MMP-2, was measured in archived BAL and serum samples from subjects in the current ozone study using Novex Zymogram gels with 0.1% gelatin as a substrate (ThermoFisher Scientific, Inc., MA, USA) according to manufacturer instructions along with 1 ng of recombinant MMP-9 or MMP-2 control (R&D System, MN, USA), followed by staining with Coomassie Blue. The signals for pro- and active MMP-9 and MMP-2 were visualized using FluorChem FC2 System (Cell Biosciences, Santa Clara, CA, USA), and the optical density was measured using ImageJ software (version 1.44, NIH, MD, USA) by two separate blinded observers, averaged, adjusted for control, and then analyzed by regression modeling using STATA 12.0 software (STATA Corp, TX, USA). Measurement of other MMPs’ activity was not performed.

RESULTS

The comparisons of DEGs from BAL cells of healthy volunteers after ozone exposure to those of various cells from patients in CVD studies revealed a set of common Gene Ontology (GO) terms involved in atherosclerotic processes.

The NextBio search strategy revealed 11 CVD studies that were relevant to atherosclerosis and coronary artery disease (Table 1). The meta-comparisons using NextBio identified a set of common DEGs between ozone and CVD studies with most DEGs regulated in the same direction (concordant correlation) (Table 2). NextBio also identified a set of GO terms in common between each of the 11 CVD studies and ozone study ranked by “Bioscore”, 31 of which were present in all 11 CVD studies (Supplemental Table S1). Many of the GO terms identified are known to contribute to atherosclerotic processes, such as cholesterol metabolism dysfunction, increased monocyte adherence, endothelial cell lesions, and matrix remodeling. Using the NextBio Disease Atlas, subgroups of enriched genes involved in ozone and CVD studies were shown to be differentially regulated in many disease phenotypes including “heart failure”, “ischemia”, “high fat diet”, and “atherosclerotic occlusive disease” (Figure 1).

IPA predicted the activation of multiple pathways involved in inflammatory cell signaling, autonomic regulation, lipid metabolism, and cell survival in common between the ozone and CVD studies.

Using IPA Knowledge base (ranked by “z-score”), eight pathways were predicted to be commonly activated between ozone and a minimum of 4 and maximum of 6 CVD studies (Table 3). These included pathways involved in inflammatory cell signaling (IL-6, IL-8, NF-κB, TREM-1 signaling), autonomic regulation (c-AMP mediated signaling), lipid metabolism (PPAR signaling), and cell survival (colorectal cancer metastasis signaling), with most commonly activated pathways being the IL-6, IL-8, and NF-κB signaling. Many of the DEGs in the ozone study were upstream molecules while those farther downstream were differentially expressed in the CVD studies.

The active form of MMP-9, the protein from one of the common DEGs between ozone and CVD studies, showed increased activity in both BAL and peripheral blood after ozone exposure.

Several matrix metalloproteinase (MMP) genes including MMP-2, MMP-8, MMP-9, MMP-12, and MMP-25 were upregulated in BAL cells of healthy adults with increasing ozone exposure, although only MMP-9 expression showed concordant correlation across commonly regulated pathways between ozone and CVD studies. As a proof-of-concept for the presumption that local airway inflammation could propagate systemically, we measured gelatinase activity of MMP-9 in archived BAL and serum samples from subjects in the ozone study. Ozone exposure increased the enzymatic activity of active form of MMP-9 in BAL samples from subjects in ozone study in a dose-dependent manner (Figure 1). Remarkably, ozone exposure also increased the activity of active MMP-9 in their peripheral blood samples in a dose-dependent manner (parameter estimate [PE]±standard error of mean [SEM] of 8.6±4.0 [p=0.032] and 4.1±2.0 [p=0.042] ng/ml increase in BAL and serum active MMP-9 per 100 ppb increase in ozone exposure, respectively) (Figure 1). There was a weak

and non-significant correlation between the activity levels of active MMP-9 in BAL and serum (r=0.24; p=0.45). The latent form of the protein (pro-MMP-9) did not show any significant change across ozone exposures. Similar assessment of MMP-2 enzymatic activity showed a dose-dependent increase in its latent form (pro-MMP-2) in BAL (PE±SEM of 0.03±0.01 [p=0.018] ng/ml increase in BAL per 100 ppb increase in ozone exposure), but no significant change in serum. No signal for active MMP-2 was observed in either BAL or serum samples.

DISCUSSION

In this study, we aimed to explore novel biological processes underlying the link between air pollution and atherosclerosis via identification of differentially regulated pathways that are in common between airway immune cells of healthy volunteers after exposure to ozone and various samples from patients with atherosclerotic CVD through a meta-analysis approach by NextBio and then IPA programs. We identified several similarly regulated pathways including inflammatory cell signaling (IL-6, IL-8, NF-κB, and TREM-1 signaling), autonomic regulation (c-AMP mediated signaling), lipid metabolism (PPAR signaling), and cell survival (colorectal cancer metastasis signaling) that could potentially be involved in adverse cardiovascular effects of ozone inhalation (Figure 3). To increase our confidence in the relevance of those pathways, we searched for and confirmed that in several of these pathways, the ozone-induced DEGs were located upstream from DEGs induced in CVD studies, which led us to make speculations about the causative role of these pathways in cardiovascular health effects of ozone inhalation. This is remarkable because although this genomic correlational study does not provide any direct link between the ozone-induced airway inflammation and the biological processes involved in atherogenesis, through this rationale, it does implicate several mechanisms via which inhalation of ozone may contribute to atherosclerotic heart disease.

Several recent animal studies have examined the relationship between ozone and cardiovascular disease risk. Controlled ozone exposure studies in rats clearly demonstrate endothelial dysfunction and reduced acetylcholine-mediated vasodilatory response in coronary vessels 24 hours following exposure to ozone (Paffett et al. 2015, Robertson et al. 2013). Other similar studies report vascular dysfunction, adverse cardiac effects, and metabolic dysfunction in response to ozone inhalation in animals (Chuang, et al. 2009, Farraj et al. 2012, Wagner et al. 2014). The molecular mechanisms underlying the pollutant-induced cardiovascular disease have also been studied in animal models, some of which have identified molecules such as endothelin-1, nitric oxide synthase, MMP-2, and MMP-9 that have been previously implicated in atherosclerosis heart disease (Kodavanti et al. 2011, Lund et al. 2007, Lund et al. 2009). However, specific molecular mechanisms underlying the effects of ozone on cardiovascular disease have not been extensively studied in humans.

Most published human studies investigating the underlying mechanisms linking air pollution to CVD mortality have limited their examination to biomarkers such as hypertension (increased adrenergic response), heart rate variability (autonomic imbalance), C-reactive protein (systemic inflammation), and fibrinogen and tissue plasminogen activator (coagulopathy), which are considered traditional risk factors for CVD (Arjomandi, et al.

2015, Devlin, et al. 2012, Donaldson et al. 2001, Li et al. 2012). Other studies have examined new biomarkers such as flow-mediated dilatation of brachial artery that underlies vascular dysfunction (Inoue et al. 2008, Kao et al. 2003). In this study, we pursued an unconventional approach to discover novel mechanisms and biomarkers involved in atherosclerosis by exploration of common molecular pathways induced by inhalation of pollutants, specifically ozone, and those that are activated in atherosclerotic heart disease. Ozone inhalation is known to cause a relatively rapid recruitment of granulocytes followed by an influx of macrophages into airways. Recruitment and activation of these inflammatory cells are thought to be mediated at least in part by release of chemokines and cytokines from the resident airway inflammatory cells, primarily composed of alveolar macrophages. Although evidence exists to support systemic dissemination of the local airway inflammation (Brook et al. 2002, Chuang, et al. 2009, Pryor et al. 1995, Robertson, et al. 2013), our study is novel in that it provides further insight into potential molecular mechanisms involved in the process of systemic propagation of local inflammation.

The ozone transcriptomics data that were analyzed in this study were obtained from total BAL cell population rather than single cell types as described previously (Leroy et al 2015). Although the study of single cell type transcriptomics could provide important information on the changes induced by ozone in each cell type, analysis of the gene expression of total BAL cells may better inform the overall effects of ozone inhalation on the lung environment. Nevertheless, cell population counts were examined in our previous study to illuminate any significant role of the BAL cell composition that may confound changes in gene expression. While ozone exposure did cause a significant neutrophilia in BAL as expected, stratification of the gene expression data did not show any significant difference in transcriptomics results between subjects with high or low neutrophilic response (Leroy, et al. 2015). Here, our interest was to focus on how ozone exposure affects the lung environment as a whole, and how the overall response to ozone in lungs may lead to systemic inflammatory effects.

Macrophages are considered as major players in the development and progression of atherosclerotic plaques. This is of interest as the most commonly activated pathways after ozone inhalation were the IL-6, IL-8, and NF-κB signaling. These pathways are involved in the recruitment and activation of monocytes and neutrophils. It is possible that these locally generated signals can indeed affect the circulating leukocytes as they pass through the pulmonary circulation and cause a systemic inflammatory response. The systemic response may subsequently result in extravasation of affected cells and their infiltration throughout micro vessels in the vascular walls and thereby actively promoting plaque progression.

Another important process in atherosclerosis is smooth-muscle cell migration from media to the intima layer of arterial walls. Matrix metalloproteinases (MMPs) are a large family of enzymes that degrade extracellular matrix such as collagen and elastin. Under the regulation of inflammatory signals, MMPs mediate changes in vascular architecture by contributing to smooth muscle cell migration within a vessel (Chen et al. 2013, Khokha et al. 2013, Page-McCaw et al. 2007, Zempo et al. 1996). Soluble MMPs are now considered potential biomarkers in delineating cardiovascular risk for plaque rupture in coronary artery disease (Liu et al. 2006). While MMPs can be secreted by monocyte and neutrophils, they may also contribute to infiltration of vascular wall by these cells. Several studies have shown a role for

MMPs in vascular wall remodeling and atherosclerosis (Khokha, et al. 2013, Page-McCaw, et al. 2007, Vacek et al. 2015, Watanabe and Ikeda 2004). Ozone exposure causes upregulation of several MMPs including MMP-9 (Leroy, et al. 2015), which in our study was the only MMP with concordant correlation across pathways commonly regulated in ozone and CVD studies. As a proof of concept, we measured the activity of MMP-9, which correlates with its protein concentration (Heo, et al. 2014), in archived BAL and serum samples from subjects after inhalation of ozone and found a dose-dependent increase in both BAL and serum, suggesting a systemic translation of the intraluminal airway production of MMP-9. This release of MMP-9 into pulmonary and systemic circulation may then contribute to pathologic arterial remodeling in vessels affected by atherosclerosis. Arterial remodeling in vessels with atherosclerotic plaques can induce shear stress and rupture of the vulnerable plaques, which ultimately could result in arterial thrombosis.

Our study has several limitations. First and foremost, we did not establish any link between the similarly regulated pathways in ozone and CVD studies. Due to the nature of our study, we did not directly link ozone exposure with causal substrates of atherosclerotic disease such as intima-media-thickness, atherosclerotic plaque, or clinical manifestations of CVD. However, our unconventional and unique approach in this exploratory study was to provide insight into potential molecular mechanisms by which ozone inhalation may exert its effects on CVD mortality. While the study itself does not provide evidence that the links exist, it does provide hypotheses on which future studies on the cardiovascular effects of ozone inhalation should focus on. For example, functional assays on peripheral blood monocytes ability to adhere to endothelial cells and their migration capability may be of more interest as opposed to the concentration of C-reactive protein. Second, from theoretical standpoint, it may be difficult to perceive that inhaled ozone that produces lung inflammation may indeed result in measureable systemic changes. However, our proof-of-concept measurement of BAL and serum (drawn by peripheral phlebotomy) levels of MMP-9 shows that inhalation of ozone even in the ambient range examined does cause measureable systemic effect. Third, since the etiology of cardiovascular disease is multifactorial, gene expression profiles of different etiologies of CVD could significantly vary from one another. Within our experimental setup, we used a broad definition of CVD since our study was exploratory in nature. However, more focused comparisons of ozone transcriptomics with that of CVD from specific etiologies may provide further mechanistic input. Lastly, the subjects in the ozone study were young healthy adult with no history of cardiovascular disease, whose response may be different than patients with coronary artery disease who are also usually older. Controlled human exposure studies are limited by ethical considerations, and while the exact relevant target population may not be possible to test, these human studies still could provide important pertinent information.

Conclusion

In conclusion, we performed a correlational study and compared transcriptomics of airway inflammatory cells obtained by BAL from healthy volunteers after exposure to high ambient levels of ozone with that of various cell types such as peripheral blood monocytes and endothelial cells of atherosclerotic plaques from patients with atherosclerotic CVD. This analysis revealed commonly regulated processes and potential molecular mechanisms by

which ozone inhalation may contribute to progression of pre-existent atherosclerotic lesions including those involved in leukocyte recruitment and activation and arterial wall remodeling. Awareness of these mechanisms could be useful in future studies focusing on the effect of air pollutants on cardiovascular health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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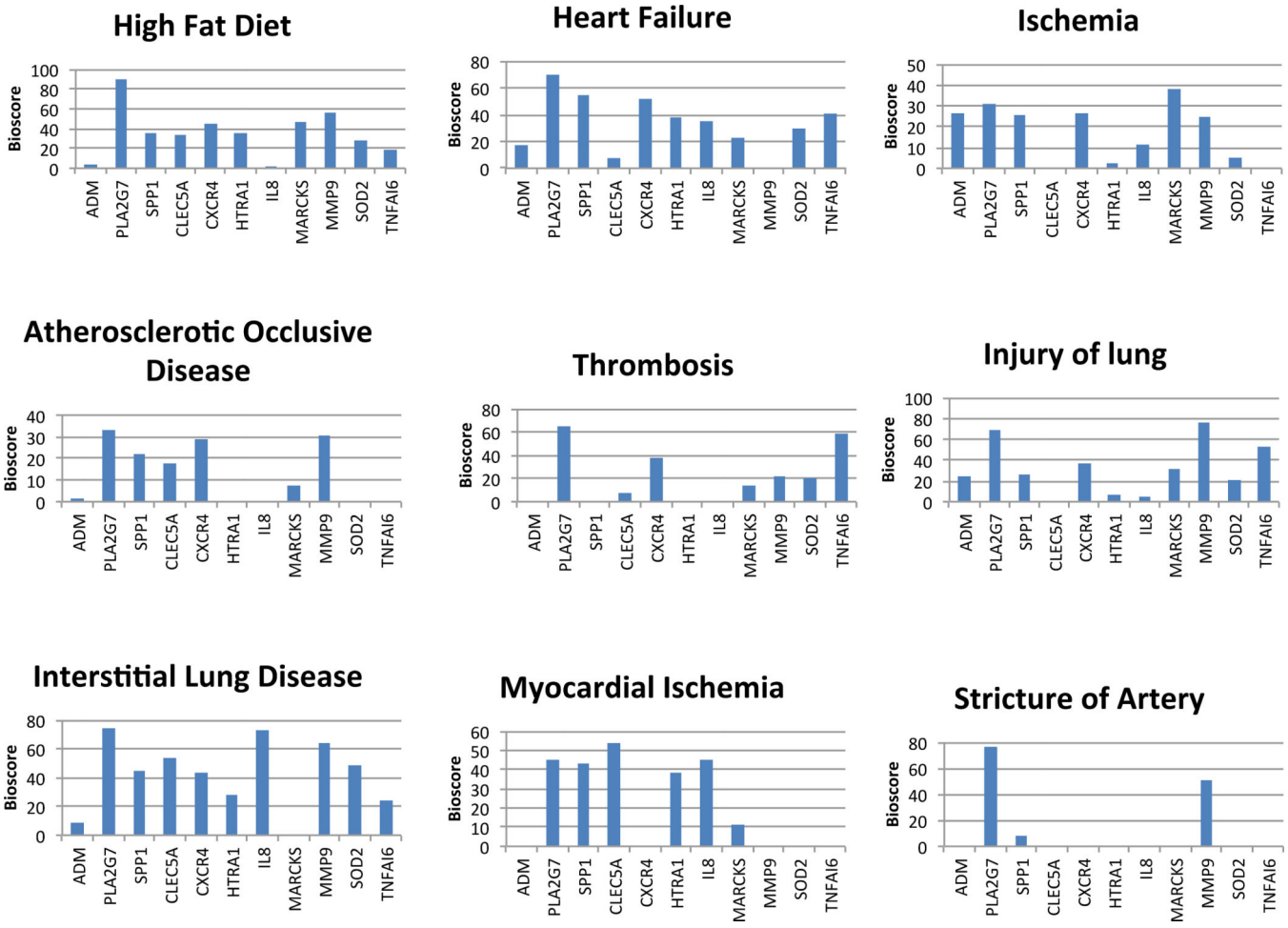

Figure 1–. Disease Processes Associated with Differentially Expressed Genes in Common between Ozone and Cardiovascular Studies.

The common differentially expressed genes were involved with a number of disease processes as shown below. Bars represent the bioscore (a ranking score from a minimum of 0 to a maximum of 100) generated by NextBio for a set of common differentially expressed genes in correlational study between the transcriptomics of ozone exposure and cardiovascular disease studies (see Methods for details).

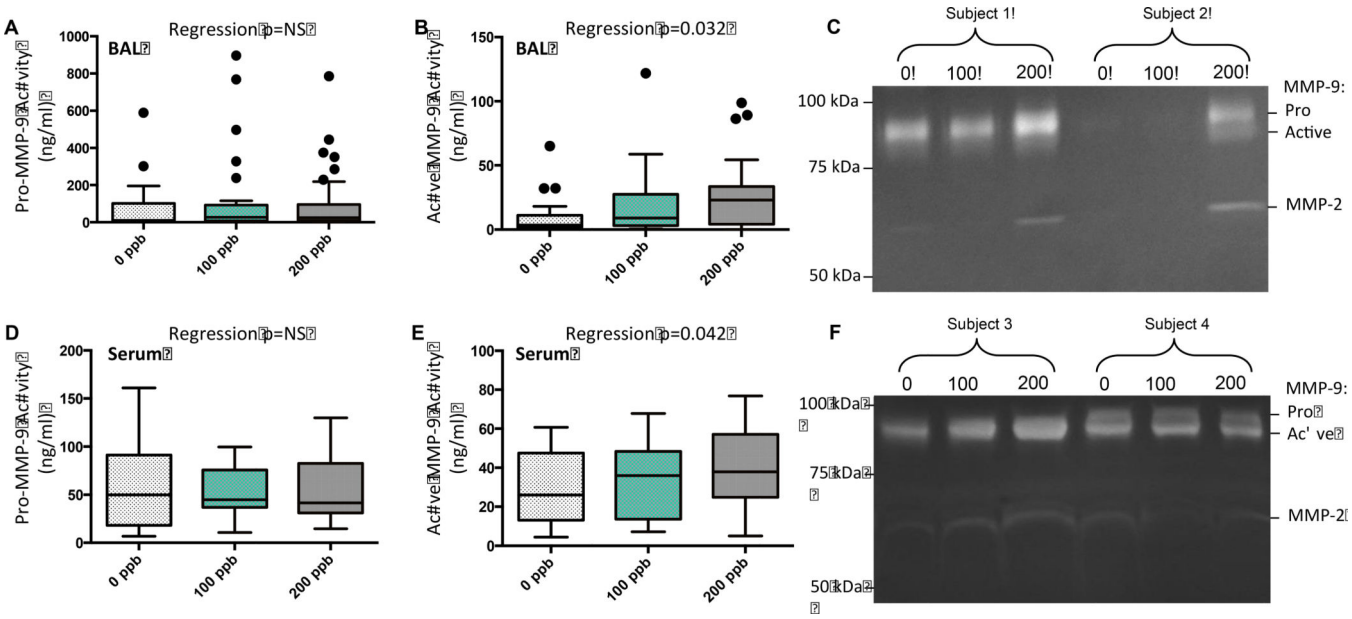

Figure 2–. Zymograms of MMP-9 Protein Activity in Bronchoalveolar Lavage and Blood after Ozone Inhalation.

Densitometry analysis of bronchoalveolar lavage fluid (BAL; Panels **A**, **B**, & **C**) and blood (serum; Panels **D**, **E**, & **F**) zymograms showed increased hydrolytic activity of the active form of MMP-9 with inhalation of increasing levels of ozone (parameter estimate±SEM of 8.6±4.0 (p=0.032) and 4.1±2.0 (p=0.042) ng/ml increase in BAL and serum active MMP-9 per 100 ppb increase in ozone exposure, respectively). The p-values are from regression modeling of MMP-9 concentration versus ozone exposure dose (NS= not significant). The box and the horizontal line represent the interquartile range and median, the whiskers represent the minimum and maximum non-outlier values, and the black circles represent outlier values.

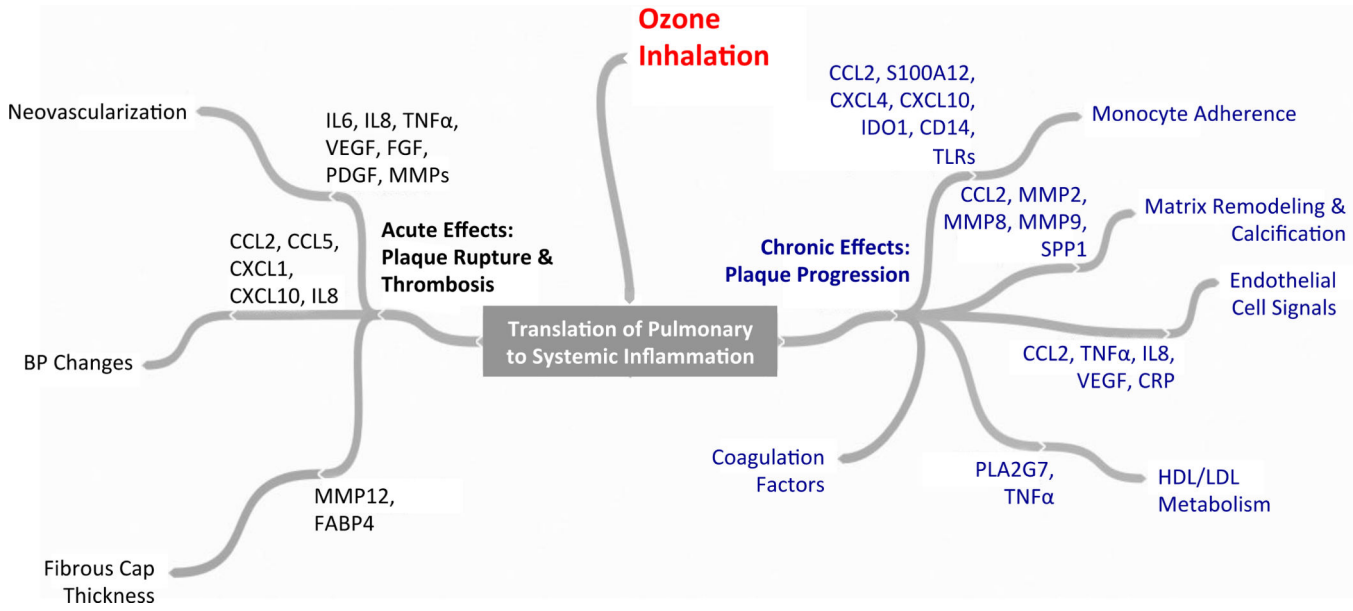

Figure 3–. Proposed Mechanisms for Effect of Ozone Inhalation on Atherosclerotic Cardiovascular Disease.

Ozone inhalation causes airway inflammation that results in systemic inflammation, which in turn contributes to the acute and chronic processes in atherosclerotic heart disease through differentially expressed genes and activation of several biological pathways.

Table 1–

Summary of the 11 studies used for meta-analysis comparison with ozone exposure gene expression study.

Rows shaded in lighter grey depict studies with samples obtained from more advanced atherosclerosis (>70% stenosis, ruptured fibrous cap, acute myocardial infarction). Darker grey rows depict studies with samples obtained from more chronic disease (coronary artery disease [CAD], acute coronary syndrome [ACS], peripheral arterial disease [PAD]). N= sample size; GSE= NCBI GEO Repository ID, PBMC= Peripheral Blood Mononuclear Cells.

CVD Study Description	CVD Study Abbreviation	Sampling location	Disease Acuity	# of Subjects	Biological Sample Studied	GSE
Patients with CAD undergoing lifestyle intervention (N=18) vs. healthy controls (N=18)	CAD_Lifestyle	Systemic	Chronic	36	Blood	66175
Patients with PAD (N=19) vs. healthy controls (N=18)	PAD_Mononuclear	Systemic	Chronic	37	PBMC	27034
Patients with CAD: Macrophages stimulated with LPS (N=11) vs. CD34+ stem cells (N=11)	CAD_LPS	Systemic	Chronic	22	PBMC	9820
Blood expression profiling in CAD pts: >70% stenosis 1 artery or 50% in 2 (N=87) vs. <25% stenosis controls (N=49)	CAD_Stenosis	Systemic	Advanced Atherosclerosis	136	Blood	20686
CD146+ cells from blood of acute myocardial infarction (N=49) vs. healthy controls (N=50)	AMI_Endothelial	Systemic		99	Endothelial cells	66360
Blood from acute myocardial infarction patients (N=4) who died during 6month follow-up vs. no AMI (N=45)	AMI_Blood	Systemic		49	Blood	34198
Atherosclerotic aortic wall of CAD patients (N=40) vs. non-atherosclerotic internal mammary artery (N=40)	CAD_Aorta	Focal	Chronic	80	Aortic Wall tissue IMA tissue	40231
Calcified stenotic aortic valves (N=5) vs. normal aortic valves (N=5)	Calcified_Aorta	Focal	Chronic	10	Aortic Valve Tissue	12644
Leukocytes from thrombi of ACS patients (N=4) vs. PBMC of healthy patients (N=4)	ACS_Leukoctyes	Focal	Advanced Atherosclerosis	8	PBMC	19339
Macrophages of carotid atheromas- ruptured thin fibrous cap(N=5) vs. stable thick fibrous cap (N=4)	Atheromas_Fibrous_Cap	Focal		9	Atheromatous Rich Plaques	41571
Plaques of patients undergoing carotid endarterectomy with future ischemic event-3.5yrs (N=25) vs. no future events-3.5yrs (N=101)	Ischemia_Endarterectomy	Focal		126	PBMC	21545

Table 2–

Overlapping Genes from NextBio Metacomparisons between Ozone and Cardiovascular Disease Studies.

Concordant correlation: differentially expressed genes (DEGs) that were similarly up- or down-regulated in both the ozone study and the compared cardiovascular study (CVD) studies; discordant correlation: DEGs that were in common but were regulated in opposite directions between the ozone study and the compared CVD studies. P-values are expressed in scientific notation for numbers with the number after E representing the power of base 10.

CVD Study	Total Number of DEGs in CVD Study	Number of DEGs in Common with Ozone Study		Concordant Correlation p-value	Discordant Correlation p-value
		Concordant Correlation	Discordant Correlation		
CAD_Lifestyle	478	2	32	7.8E-3	1.4E-14
PAD_Mononuclear	1497	27	2	3.6E-12	3.1E-2
CAD_LPS	11459	147	49	2.0E-50	2.9E-2
CAD_Stenosis	226	13	2	1.8E-9	6.6E-2
AMI_Endothelial	7332	106	42	4.1E-48	0.01.5E-2
AMI_Blood	1941	27	25	5.8E-21	1.0E-3
CAD_Aorta	6910	90	35	5.2E-16	8.6E-3
Calcified Aorta	5033	80	0	1.6E-29	-
ACS_Leukocytes	5796	45	4	9.8E-19	3.9E-3
Atheromas_FibrousCap	106489	51	3	4.7E-9	4.5E-2
Ischemia_Endarterectomy	132	2	0	3.1E-3	-

Table 3–

Predicted pathway activation among the Ozone and CVD studies.

Ingenuity pathway software (IPA) scores each pathway based on p-value (IPA Score=-log[p-value]). An IPA Score of >1.3 represents a statistically significant pathway (p-value<0.05). Percent Activity represents the ratio of differentially expressed genes of molecules (defined as having a fold change of |1.2| and a p-value <0.05). Z-score is the predicted activation value of the pathway by IPA. Studies are ordered with ozone first followed by the CVD studies that are predicted to be most active according to their z-score.

Canonical Pathway	Study Abbreviation	IPA Score	Percent Activity	Z-score	Active Molecules
IL-8 Signaling	Ozone	4.29	6.01%	3.32	PLD4,CXCL8,CCND2,FLT1,CXCR2,GNG2,MMP2,CXCR1,MMP9,ITGB3,PRKCB
	AMI_Endothelial	4.98	6.52%	3.46	VEGFA,CXCL8,FOS,ICAM1,JUN,HBEGF,CXCL1,GNA13,IRAK3,PDGFC,MMP9, ITGAX
	PAD_Mononuclear	1.93	3.80%	2.65	CXCL8,FOS,ICAM1,ANGPT1,RHOU,PTGS2,MAP4K4
	ACS_Leukoctyes	2.52	4.35%	2.12	VEGFA,HMOX1,ICAM1,FLT1,ITGAV,IL9,ATM,IRAK2
	Calcified_Aorta	4.34	5.98%	2.12	RAB11FIP2,GNAI2,PLD3,RHOG,MAP2K2,GNB2,FIGF,PIK3R2,NFKBIB,MAP4K4, LIMK1
	CAD_LPS	1.85	3.80%	1.89	ITGB2,PLD3,NRAS,MRAS,PIK3R5,GNG5,PRKCZ
	CAD_Lifestyle	3.65	5.43%	2.53	VEGFA,HMOX1,GNAI3,CCND2,RHOB,DEFA3 (includes others),CHUK,IRAK3,MMP9,AZU1
IL-6 Signaling	Ozone	4.47	7.76%	3.00	IL1R2,CXCL8,TNFAIP6,IL1RN,IL6R,IL1R1,TNFRSF1B,A2M,IL18RAP
	Calcified_Aorta	3.73	6.90%	2.65	COL1A1,SHC1,MAP2K2,TNFAIP6,CD14,PIK3R2,NFKBIB,MAP4K4
	CAD_LPS	1.65	4.31%	2.24	IL1R2,NRAS,MRAS,PIK3R5,CEBPB
	CAD_Lifestyle	3.72	6.90%	2.12	VEGFA,IL1R2,IL18,TNFAIP6,IL1R1,CHUK,JAK2,MCL1
	AMI_Endothelial	7.11	10.30%	2.11	VEGFA,IL1R2,CXCL8,SOCS1,SOCS3,FOS,IL1A,JUN,NFKBIA,IL1RN,CD14,IL1B
	PAD_Mononuclear	2.34	5.17%	1.63	CXCL8,SOCS3,FOS,NFKBIA,MAP4K4,MCL1
NF-κB Signaling	Ozone	3.23	5.33%	3.00	TLR2,IL1R2,TLR10,FLT1,IL1RN,TLR6,IL1R1,TNFRSF1B,PRKCB
	AMI_Endothelial	5.28	6.98%	2.31	TLR2,IL1R2,TLR4,IL1A,NFKBIA,IL1RN,PELI1,FCER1G,IL1B,MAP3K8,LTBR,IRAK3
	CAD_LPS	2.00	4.07%	1.89	IL1R2,NRAS,TLR8,MRAS,PIK3R5,IGF2R,PRKCZ
	CAD_Lifestyle	3.89	5.81%	1.67	TANK,IL1R2,TLR4,IL18,PELI1,TLR8,IL1R1,CHUK,IRAK3,TNFRSF17
	PAD_Mononuclear	1.10	2.91%	0.45	TLR4,NFKBIA,TLR8,TRAF5,MAP4K4
	ACS_Leukoctyes	1.12	2.91%	−0.45	TNIP1,FLT1,NFKBIE,GSK3B,ATM
Dendritic Cell Maturation	Ozone	3.23	5.33%	2.12	TLR2,IL1RN,CD1A,HLA-DMA,CD1B,FCGR2B,TNFRSF1B,CD1C,CCR7
	AMI_Endothelial	5.92	7.34%	3.47	TLR2,TLR4,FCGR2C,IL1A,NFKBIA,ICAM1,IL1RN,FCGR2A,FCER1G,IL1B,CD83,LTBR,CREB5
	Calcified_Aorta	1.03	2.82%	2.24	COL1A1,FSCN1,CREB1,PIK3R2,NFKBIB

Canonical Pathway	Study Abbreviation	IPA Score	Percent Activity	Z-score	Active Molecules
	CAD_Lifestyle	1.96	3.95%	1.13	TLR4,CD1D,IL18,IGHG3,LTB,CHUK,JAK2
	ACS_Leukoctyes	1.08	2.82%	0.45	ICAM1,NFKBIE,CD83,STAT2,ATM
	CAD_Stenosis	1.29	2.26%	−2.00	IGHG4,IGHG1,CREB5,IGHG2
TREM1 Signaling	Ozone	5.26	11.40%	2.83	TLR2, CXCL8, TLR10, NLRP3, CCL2, TLR6, FCGR2B, CCL3
	AMI_Endothelial	7.07	13.30%	3.16	TLR2, CXCL8, TLR4, TREM1, ICAM1, NLRP3, IL1B, CD83, CCL3, ITGAX
	PAD_Mononuclear	2.49	6.67%	2.24	CXCL8, TLR4, TREM1, ICAM1, TLR8
	ACS_Leukoctyes	2.52	6.67%	2.24	CXCL3, ICAM1, CCL2, CD83, CCL7
	CAD_Lifestyle	1.71	5.33%	2.00	TLR4, IL18, TLR8, JAK2
PPAR Signaling	Ozone	2.08	5.56%	−2.24	IL1R2, IL1RN, IL1R1, TNFRSF1B, IL18RAP
	PAD_Mononuclear	1.45	4.30%	−2.00	FOS, NFKBIA, PTGS2, MAP4K4
	Calcified_Aorta	1.42	4.30%	−2.00	SHC1, MAP2K2, NFKBIB, MAP4K4
	CAD_Lifestyle	1.42	4.30%	−2.00	IL1R2, IL18, IL1R1, CHUK
	AMI_Endothelial	4.34	8.60%	−2.83	IL1R2, FOS, IL1A, JUN, NFKBIA, IL1RN, IL1B, PDGFC
cAMP-mediated Signaling	Ozone	1.51	3.24%	1.89	HRH2, P2RY14, CAMK1, CCR4, CXCR2, DUSP6, ADORA3
	AMI_Endothelial	2.44	4.11%	1.67	P2RY13, HCAR3, TULP2, DUSP1, CREM, FPR2, PDE4B, CREB5, FPR1
	CAD_LPS	1.95	3.65%	−0.38	LAMTOR3, CAMK1, NPR3, PDE4A, ADCY6, CREB3L4, PKIA, PDE6B
	CAD_Stenosis	1.02	1.83%	−1.00	GPER1, PDE9A, PDE1A, CREB5
	CAD_Aorta	2.50	4.11%	−1.67	GPER1, NPY1R, PDE10A, PDE3A, PDE8B, PDE4D, RAP1A, AGTR1, PDE1C
Colorectal Cancer Metastasis Signaling	Ozone	3.41	4.76%	3.32	TLR2, TLR10, TCF4, IL6R, GNG2, TLR6, MMP2, MMP12, JAK3, MMP25, MMP9
	CAD_Lifestyle	2.81	4.24%	3.16	VEGFA, TLR4, RHOB, MMP8, TLR8, IFNGR1, JAK2, MMP9, TCF7L2, PRKAR1A
	AMI_Endothelial	2.23	3.81%	3.00	VEGFA, TLR2, TLR4, FOS, JUN, IFNGR1, MMP25, PDGFC, MMP9
	PAD_Mononuclear	1.02	2.54%	2.45	TLR4, FOS, TLR8, RHOU, PTGER2, PTGS2
	ACS_Leukoctyes	1.44	2.97%	0.38	VEGFA, IL6ST, JAK1, FZD5, GSK3B, FZD7, ATM

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