Acute Ozone-Induced Differential Gene Expression Profiles in Rat Lung

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Ozone (O₃) is an oxidant gas that can directly induce lung injury. Knowledge of the initial molecular events of the acute O₃ response was useful in developing biomarkers of exposure or response. Toward this goal, we exposed rats to toxic concentrations of O₃ (2 and 5 ppm) for 2 hr and the molecular changes were assessed in lung tissue 2 hr postexposure using a rat cDNA expression array containing 588 characterized genes. Gene array analysis indicated differential expression in almost equal numbers of genes for the two exposure groups: 62 at 2 ppm and 57 at 5 ppm. Most of these genes were common to both exposure groups, suggesting common roles in the initial toxicity response. However, we also identified the induction of nine genes specific to 2-ppm (thyroid hormone-β receptor c-erb-A-β and glutathione reductase) or 5-ppm exposure groups (c-jun, induced nitric oxide synthase, macrophage inflammatory protein-2, and heat shock protein 27). Injury markers in bronchoalveolar lavage fluid (BALF) were used to assess immediate toxicity and inflammation in rats similarly exposed. At 2 ppm, injury was marked by significant increases in BALF total protein, N-acetylglucosaminidase, and lavageable ciliated cells. Because infiltration of neutrophils was observed only at the higher 5 ppm concentration, the distinctive genes suggested a potential amplification role for inflammation in the gene profile. Although the specific gene interactions remain unclear, this is the first report indicating a dose-dependent direct and immediate induction of gene expression that may be separate from those genes involved in inflammation after acute O₃ exposure.

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The photochemical oxidant ozone (O₃) is the air pollutant in smog thought to be of greatest concern with regard to acute health effects [U.S. Environmental Protection Agency (EPA) 1996]. Although considerable progress has been made in improving U.S. air quality since air pollution standards were established in 1970, about 50% of the U.S. population currently lives where O₃ levels exceed the National Ambient Air Quality Standard (NAAQS) (U.S. EPA 1993). Of the six NAAQS pollutants, O₃ has been the most problematic pollutant to control because it is formed from intermediates originating from many different sources. Hence, concerns about adverse health impacts remain. It is known that acute exposure to this gas at ambient levels results in acute lung injury and inflammation in humans (Devlin et al. 1991). Airway epithelial cells are damaged and lung function is impaired in both humans and laboratory animals (Hatch et al. 1994; Koren et al. 1989). Additionally, because O₃ reaches the deep lung and damages distal airway and proximal alveolar structures (including the surface epithelia and connective tissues), there is a potential for permanent damage with repeated exposure and injury to the deep lung (Costa et al. 1985). Recent epidemiological studies have associated increased morbidity, particularly in children with asthma, during periods of high O₃ pollution (Tolbert et al. 2000; White et al. 1994).

O₃ appears to induce initial damage to the respiratory epithelium because of an oxidative cascade after its initial reaction with lipids and proteins at the air–liquid interface (Pryor 1992). Injury to the epithelium results in sloughing of ciliated cells into bronchoalveolar lavage fluid (BALF). Increased protein concentration and N-acetylglucosaminidase (NAG) activity in the BALF also occur because of leakage of proteins from blood plasma or intracellular spaces (Dye et al. 1999; Hu et al. 1982; Vincent et al. 1996). The release of inflammatory cytokines and chemokines from injured cells initiates the infiltration of neutrophils, which are also increased in the BALF (Devlin et al. 1991) and at least in the short run are thought to contribute to injury. Despite the evidence that this overt process wanes when repeated over time, it appears that the injury and inflammation cascade promotes cellular hypertrophy and the deposition of interstitial matrix materials and generalized remodeling of the fine structures of the deep lung (Chang et al. 1992; U.S. EPA 1993).

O₃ is also hypothesized to initiate intracellular oxidative stress through ozoneide and hydroperoxide formation (Pryor 1992). These intracellular oxidants are likely to activate gene transcription through redox-mediated signaling pathways that govern the cascade of injury, repair, and other cellular responses associated with the oxidant burden. For example, the inflammatory cytokines and chemokines interleukin (IL-8), macrophage inflammatory protein-2 (MIP-2), and cytokine-induced neutrophil chemoattractant (CINC), which are found in the BALF and lung tissues of rodents exposed to O₃ (Michele et al. 2002; Zhao et al. 1998), can initiate differential transcriptional activation of genes. Because gene expression is mediated by various transcription factors, which can ultimately determine the outcomes of the challenge, we hypothesized that gene expression profiles derived using gene arrays could aid in identifying exposure-specific gene regulation for O₃ that might then lead to the identification of potential gene markers for acute lung injury. Although the inflammatory response to O₃ has been well documented, the earliest signaling pathways associated with this process are not known.

The acute O₃ lung injury model has been widely used to explore injury and repair processes (Bassett et al. 1988; Klebeberger et al. 1997; Prows et al. 1999). It provides a well-documented and reproducible tool to study the fundamental events associated with acute lung injury induced by oxidant overload. It was felt that oxidant-based profiles arising from this study might aid in our understanding of various biochemical pathways involved in lung injury, inflammation, and repair processes. It may also be possible to identify acute markers associated with long-term outcomes that serve to guide hypotheses generation to explore further understanding of acute lung injury.

Commercially available microarray technologies can facilitate efforts at global gene expression profiling. However, the rat genome is not yet completely sequenced, and the global approach with microarrays containing numerous expressed sequence tags may not be able to provide the needed information on possible candidate genes that can be further explored at

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this time. We therefore used the nylon micro-
array with a limited and targeted number of
well-characterized rat genes to identify gene
expression profiles involved in the acute
response to toxic doses of O₃.

Materials and Methods

Animals. Fischer 344 rats (male, 90 days of
age) were obtained from Charles River
Laboratories (Raleigh, NC) and kept in tem-
perature- and humidity-controlled rooms with
a 12/12-hr light/dark cycle. Standard rat chow
(ProLab, Brentwood, MO) and water were
provided ad libitum. The animal facility is
Association for Accreditation of Laboratory
Animal Care approved, and all procedures
were reviewed and implemented through the
Institutional Animal Care and Use Committee
process of the U.S. EPA National Health and
Environmental Effects Research Laboratory.

Inhalation exposures. Rats (six animals per
group) were placed in individual stainless-steel
wire-mesh cages inside a 135-L exposure
chamber and exposed to either 2.0-ppm O₃ or
5.0-ppm O₃ for 2 hr. Control animals were
exposed to filtered room air. Chamber O₃ con-
centration was monitored with a Dasibi model
1003AH O₃ monitor (Dasibi Environmental
Corp., Glendale, CA).

Lung removal. Two hours postexposure,
rats were anesthetized by an ip injection of
(50 mg/kg body weight) pentobarbital
(Abbott Laboratories, North Chicago, IL)
and exsanguinated by severing the dorsal
aorta. The chest cavity was opened, and the
lungs were removed en bloc. Individual lobes
were separated, quick frozen in liquid nitro-
gen, and stored at −80°C until used for RNA
extraction.

Bronchoalveolar lavage. Rats exposed
identically to those used for gene expression
analysis were also anesthetized and bled. A
tracheal cannula was inserted to about 0.5 cm
above the carina, and the whole lung was
tracheal cannula was inserted to about 0.5 cm
frozen. The other fraction was cytocentrifuged
with a slight modification. The reaction was
extended for 15 min after the addition of cold
40 µM dATP to improve the quality of the
cDNA in a reverse transcriptase reaction fol-
lowing the manufacturer-suggested protocol,
unincorporated nucleotides using a spin col-
umn (Nucleospin extraction kit, Clontech),
unincorporated nucleotides using a spin col-
umn (Nucleospin extraction kit, Clontech),
and corrected for background with the median
setting following the protocols defined in the
AtlasImage software, version 2.7. Spot density
values for all the genes were imported to
GeneSpring software (version 6.0; Silicon
Genetics, Redwood City, CA) and subjected
to quality control (QC) measures to identify
the total number of genes that showed
hybridization signals above the background in
all 12 arrays (four arrays per group). The QC
gene list generated was analyzed to identify
altered genes using a filter of 2-fold change.

Statistical analysis. Gene lists generated
(for genes either induced or suppressed by
2-fold) were subjected to statistical analysis
using the GeneSpring preprogrammed statisti-
cal package. Genes whose expressions were
altered by 2-fold were subjected to one-way
analysis of variance (ANOVA) setting p-values
of < 0.05. The comparison is performed for
each gene in all the groups, and the genes with
the set cutoff (p-values of < 0.05) are returned.
The genes selected by one-way ANOVA were
also corrected for false rate discovery following
the Benjamini and Hochberg (1995) method.
Gene lists (induced/suppressed) generated in
this way were used in Venn diagram analysis
to identify the genes that were common or
unique to each exposure group (2 or 5 ppm)
and were listed.

Real-time reverse transcriptase PCR.
Relative gene expression was quantified using
real-time reverse transcriptase (RT) quantita-
tive PCR on selected genes to verify the
microarray data. Total RNA (5 µg) was reverse
transcribed to generate first-strand cDNA
using Moloney murine leukemia virus reverse
transcriptase (Invitrogen) and random primer
mix (Invitrogen). Taqman predeveloped assay
reagents (Applied Biosystems, Foster
City, CA) were used for amplification of
induced nitric oxide synthase (NOS2), JUN,
and glyceraldehyde-3-phosphate dehydroge-
nase (GAPDH). Oligonucleotide primer pairs
for thyroid hormone-β receptor (Thrb)
 glu-
thionine reductase (Gsr) were designed using
a primer design program (Primer Express,
Applied Biosystems) and obtained from
Integrated DNA Technologies (Coralville,
IA). Quantitative fluorogenic amplification of
cDNA was performed using the ABI Prism
7700 Sequence Detection System (Applied
Biosystems). The relative abundance of
mRNA levels was determined from standard
curves generated from a serially diluted stan-
dard pool of cDNA prepared from human
bronchial epithelial cells. The relative abun-
dance of GAPDH mRNA was used to normal-
ize levels of the mRNAs of interest.
Results
Bronchoalveolar lavage fluid analysis. The indicators for lung injury and inflammation measured in BALF 2 hr after the 2-hr exposure to air or 2 or 5 ppm O₃ are presented in Table 1. BALF protein concentrations were increased significantly by 20-fold in the 5-ppm group but were changed only about 1.5-fold in the 2-ppm group. NAG was increased 7.5-fold in the 5-ppm group and 1.5-fold in the 2-ppm group. Lysozyme was not significantly affected in either exposure group. Total cell counts appeared to be decreased by about 20% after both the 2- and 5-ppm exposures. This decrease is common to O₃-exposed animals immediately after exposure because it is thought that macrophages become activated and are not available to BAL. Neutrophil and ciliated cell percentages in the BALF (which are normally close to zero) increased significantly in both the 2- and 5-ppm groups in a concentration-dependent manner. However, this increment at 2 ppm, although significant, was in the range of BALF neutrophils considered “normal” for control rats. Had BAL been conducted 12–15 hr postexposure, as is more typical (Hatch et al. 1986), it is likely that these values would have been considerably higher. Notably, however, in the 5-ppm group, the neutrophils and ciliated cells were substantially increased to 23 and 40%, respectively, of total cells, indicative of concomitant immediate airway and alveolar damage and inflammation.

Microarray analysis. Analysis of the expression of 588 genes spotted on the rat cDNA nylon array showed that 540 genes were expressed constitutively in the lung of all the treatment groups including controls. With exposure to O₃, statistically significant augmentation (with 2-fold set as a minimal induction threshold in the statistical analysis) of expression was found in 62 genes at 2 ppm and 57 genes at 5 ppm O₃. Of these genes, 26 were induced commonly in both 2- and 5-ppm exposure groups, and a total of 36 genes in the 2-ppm group and 31 genes in the 5-ppm group were suppressed (Table 2). Despite the difference in the exposure concentration, the immediate toxic response appeared to be mediated by the transcriptional regulation of many common genes: induction of 17 and suppression of 25 genes in both exposure groups. Further analysis indicated concentration-specific induction and/or suppression of unique genes (Table 2), suggesting their possible roles in initiating different downstream signaling networks. The up-regulated genes that were common to both 2 and 5 ppm O₃ treatment are listed in Table 3; the common down-regulated genes are listed in Table 4. Induced genes unique to both the 2- and 5-ppm exposure groups are listed in Table 5. Similarly, suppressed genes that are unique to the 2- and 5-ppm exposure groups are listed in Table 6.

Of 13 functional groups represented on this microarray, O₃-altered gene expression profiles were distributed predominantly into four broad functional groups: a) metabolism (lipid, protein), b) intracellular transducers/stress response (modulators, oncogenes), c) growth factors/receptors (kinases, activators/inhibitors), and d) cell surface receptors (adhesion proteins and ligands). Among these groups, stress-responsive genes, oncogenes, and cell cycle–related genes were up-regulated, whereas cell surface receptors were down-regulated. Lipid metabolism genes were differentially expressed in response to O₃ inhalation. The altered expression in lipid metabolism and the transcription factors nuclear factor κB (Nfkb1), ras oncogenes, and insulin-like growth factor (IGF) binding protein-2 (Igfbp2) and the concentration-specific differential expression of stress-response proteins such as Jun, Gis, and calcium-dependent signal mediators, observed in the present study for the first time, will shed new light on their possible roles in acute O₃ toxicity. Further analysis of the altered expression of genes unique to 2 or 5 ppm (Tables 5, 6) will be more useful in identifying exposure-specific immediate lung injury.

To validate the altered gene expression observed in the microarray assay, real-time RT-PCR was performed on five selected genes (four of which were not known to be associated with O₃ toxicity, and one known gene was found altered in rat lung tissue on exposure to O₃). As shown in Table 7, the expression of these five genes is in good agreement with the microarray analysis.

Discussion
The studies we report here represent part of our ongoing effort to characterize the immediate biologic responses of rat lung tissue to a toxic dose of O₃, and to use this information to develop biomarkers for its toxicity (Hatch et al. 1986, 1994). This effort was to generate gene expression profiles for rat lung tissue using high-throughput microarray technologies to distinguish levels of injury based on the differential expression of specific groups of genes thought to be involved in this process. The gene expression profiles derived at 2 hr after O₃ inhalation represent toxicant-induced transcriptional activation/inactivation that is not likely confounded by other physiologic factors as might occur after established inflammation. To the best of our knowledge, our present study is the first to be published on the near-immediate impact of acute O₃ exposure on gene expression response profiles in rat lung tissue. Two related reports on O₃-altered gene expression profiles have appeared in the literature. One involved mice (Gohil et al. 2003) assayed after repeated O₃ exposures (1 ppm; 8 hr/day) for 3 days, with analysis performed immediately after the third exposure. Another investigation was carried out in rats exposed to 1 ppm O₃ for 3 hr (Bhalla et al. 2002) and evaluated for the expression of inflammatory marker genes at a relatively late time point (10–12 hr postexposure). In both studies it is likely that significant inflammation and repair processes were involved. In contrast, gene expression profiles derived in the present study represent the near-immediate transcriptional alterations in response to a single exposure to a toxic dose of O₃, and, not surprisingly, present a profile different from these other studies.

In the present study we exposed rats to 2 and 5 ppm of O₃ for 2 hr. The 2-ppm exposure was selected to represent a possible human exposure during vigorous human exercise at a high exposure concentration of approximately 0.4 ppm of O₃ (Hatch et al. 1994), whereas the higher level (5 ppm) might represent a more severe oxidant challenge that may initiate acute respiratory distress syndrome involving concomitant oxidant injury and inflammation. Using ³¹O-labeled O₃, we (Hatch et al. 1994) have shown that the impact of acute exposure to O₃ at 0.4 ppm...
with intermittent heavy exercise in humans resulted in lung tissue dosimetry approximately equal to that of the rat exposed sedentarily to 2 ppm for the same 2-hr period.

The initial interaction of O₃ with the unsaturated fatty acids in the epitheal lining fluid is thought to generate lipid ozonation products that drive various signaling cascades that result in the biochemical events characteristic of O₃ pulmonary toxicity. As such, the immediate molecular changes leading to gene induction at this step may be identifiable using high-throughput technologies leading to candidate biomarkers for O₃ exposure and toxicity. Thus, induced genes may ultimately lead to the development of markers that can be screened using noninvasive approaches (Krishna et al. 1998; Liu et al. 1999).

The airway epithelium is the first line of defense against inhaled toxins and also is the primary site of O₃-induced injury (Koren et al. 1991). Acute exposure to O₃ leads to immediate epithelial injury, pulmonary neutrophic inflammation subsequent to permeability changes, and the leakage of serum proteins into the air spaces of the lung. The increase in BALF protein content, NAG activity, and recoverable neutrophils are collectively indicative of airway and alveolar epithelial necrosis. This pattern of markers and inflammatory cellular response is typically observed at later time points (12~18-hr postexposure) as markers of exposure and injury (Bhalla and Gupta 2000; Hatch et al. 1994; van Bree et al. 2001). The earliest cellular and molecular events are generally not studied because of lack of sensitive tools.

The statistically significant differences in the expression of 119 genes in the two exposure groups together suggest that immediate transcriptional regulation of these genes may be involved in the tissue injury and/or regenerative responses. The gene expression data derived in the present study suggest that the O₃-induced injury is mediated by differential activation of genes predominantly distributed in two groups: fatty acid metabolism and cell proliferation. In contrast, genes representing signaling mediators, receptors, or second messengers were suppressed. Interestingly, the altered gene expression profiles of the two exposure groups (2 and 5 ppm) indicated that most genes affected were common (Tables 3, 4). It remains to be seen if the response generalizes to other oxidants.

The 3.5-fold induction in the expression of the adhesion molecule L-selectin observed 2 hr after exposure to 2 and 5 ppm O₃ suggests its role in the migration and increased accumulation of neutrophils observed at this early time point. Induction of other adhesion molecules, including P-selectin, has been observed in human BALF cells on acute exposure to 0.12 ppm of O₃ (Blomberg et al. 1999; Krishna and Holgate 1999). Increased expression of apurinic and apyrimidinic (AP) endonuclease (~5-fold) indicates possible activation of DNA repair processes (He et al. 2001). Simultaneous induction of β-arrestin-1 and β-arrestin-2, along with cyclins, clearly suggests the initiation of epithelial cell DNA repair and subsequent cell proliferation. Besides, β-arrestin proteins, which belong to the G-protein–coupled receptor family, are also known to act as scaffold proteins that mediate the activation of MAP kinase cascades (Lutterel et al. 2001; Sun et al. 2002).

The differential activation of lipid metabolism genes (induction of fatty acid amide hydrolase, phospholipase A₂–activating protein) agrees with the long-knowledge biochemical evidence of lipid ozonation products generated from the phospholipid pools of the pulmonary surfactant or the epithelial cell membranes (Kafoury et al. 1999). In vitro O₃ exposure also has been shown to activate phospholipase A₂, C, and D in cultured epithelial cells (Wright et al. 1994). The consequences of altered phospholipases and the generation of lipid signal transduction network elements in response to lipid ozonation products are outlined in Table 4.

### Table 3. List of common genes induced (>2-fold) in rat lung after 2-hr exposure to 2 and 5 ppm O₃.

| Accession no. | Gene symbol | Gene name | Fold change |
|---------------|-------------|-----------|-------------|
| U72497        | Faah        | fatty acid amid hydrolase | 14.17       |
| M52848        | Cecam1      | ecp-ATPase precursor (Cell-CAM105) | 10.00       |
| U17901        | Piaa        | phospholipase A-2 activating protein (PLAP) | 7.96        |
| U90973        | Kras2       | K-RAS 2B proto-oncogene | 7.43        |
| D14015        | Ccne1       | G1/S specific cyclin (cyclin E1) | 5.57        |
| L07736        | Cpt1a       | mitochondrial carnitine (3-palmitoyltransferase | 5.43        |
| D10728        | Cds5        | T-cell surface glycoprotein (lymphocyte antigen CD5) | 4.89        |
| D44495        | Apex1       | aurupinic/apyrimidinic endonuclease | 4.86        |
| X17372        | Ldhr        | low-density lipprotein receptor | 4.61        |
| AF007789      | Plaur       | uronine receptor | 4.45        |
| AF017437      | Cd47        | integrin-associated protein form 4 | 3.93        |
| M91589        | Arb1        | beta-arrestin 1 | 3.80        |
| D10831        | Sell        | L-selectin precursor | 3.50        |
| X98490        | Rpa2        | replication protein A | 3.38        |
| M91590        | Arb2        | beta-arrestin 2 | 2.41        |
| L26276        | Nkbi1       | NF-kappa B transcription factor p105 subunit | 2.38        |
| X70871        | Ccng1       | G2/M specific cyclin G (cyclin G1) | 2.11        |

*Genes that were induced and common to both 2- and 5-ppm–exposed rat lung are listed here. *Accession numbers derived from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/). *Gene symbols and names derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). *Fold induction in gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).

### Table 4. List of common genes suppressed (>2-fold) in rat lung after 2 hr exposure to 2 and 5 ppm O₃.

| Accession no. | Gene symbol | Gene name | Fold change |
|---------------|-------------|-----------|-------------|
| U87306        | Unc5b       | transmembrane receptor UNC5H2 | -33.3       |
| J04486        | Igfbp2      | insulin like growth factor binding protein-2 (IGFBP-2) | -15.5 (2 ppm) |
| D26495        | Cd1d1       | rat CD1 antigen precursor | -10.78      |
| M63334        | Camk4d      | calcium-calmodulin dependent protein kinase IV | -10.40      |
| M31833        | Tacr2       | substance K receptor | -6.42       |
| L27057        | Pde4a       | cAMP phosphodiesterase 4A | -5.14       |
| V02171        | Actb        | cytoplasmic beta-actin | -4.58       |
| X06890        | Rab4a       | ras-related protein Rab4A | -4.28       |
| U87305        | Unc5a       | transmembrane receptor UNC5H1 | -3.97       |
| M94092        | PkB         | PKI-beta cAMP protein kinase inhibitor | -3.73       |
| M94056        | Dpep1       | dipeptidase | -3.64       |
| L34067        | Gpc1        | glypican-1 precursor | -3.33       |
| X13817        | Calm3       | calmodulin | -3.21       |
| Z22867        | Pde5b       | cAMP-dependent phosphodiesterase | -3.21       |
| A3208445      | Psen2       | presenilin2 | -3.10       |
| M59895        | Maccs       | mistriostated alanine-rich C-kinase substrate | -2.93       |
| J05155        | Plcg2       | phospholipase C gamma 2 | -2.93       |
| J03754        | Atp2b2      | PMCA, calcium-transporting ATPase plasma membrane form | -2.92       |
| X06889        | Rab3a       | ras-related protein Rab3A | -2.60       |
| J08006        | Plcg1       | phospholipase C gamma 1 | -2.57       |
| U68278        | Epha3       | Eph-related receptor tyrosine kinase (Rek4) | -2.54       |
| M22748        | Lif         | leukemia inhibitory/cholinergic neuronal differentiation factor | -2.44       |
| M66025        | Vgf         | VGF nerve growth factor, inducible | -2.40       |
| U34841        | Gprk5       | G-protein-coupled receptor kinase 5 | -2.31       |
| U00659        | Stxbp1      | Sec1, syntaxin binding protein 1 | -2.11       |
| M94043        | Rab38       | RAB-related GTP-binding protein | -2.02       |

*The genes that were found down-regulated/suppressed and common to both 2- and 5-ppm–exposed rat lung are listed here. *Accession numbers derived from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/). *Gene symbols derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). *Fold suppression of gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).
complex (Kafouri et al. 1999). Lipid signal transduction networks involve cross-talk among various isoforms (Liscovitch 1992). The altered expression of genes involved in lipid metabolism suggests their possible involvement in initiating a cascade of biochemical events that can lead to cellular responses characteristic of O₃ toxicity in the lung.

The present study also indicated dose-specific unique gene expression profiles. The high dose of 5 ppm induced the expression of various stress-response genes such as the transcription factor Jun, Nos2, MIP-2 (Cxc2), and heat shock protein 27 (Hspb1). This is the first observation of such an immediate induction of these genes. Although the induced expression of heat-shock proteins MIP-2 and Nos2 has been reported at later time points such as 4–8 hr after exposure to 2 ppm O₃ (Driscoll et al. 1993; Johnston et al. 2001; Zhao et al. 1998), the induction observed here occurred within 2 hr after 2 ppm of 5 ppm but not 2 ppm. The induction of MIP-2 and Nos2 only in the rat lungs exposed to 5 ppm O₃ suggests their participation in or the result of the rapid and immediate influx of neutrophils observed in this group. Induction of Jun and Hspb1 in rat lungs exposed to 5 ppm O₃ suggests a role in downstream signaling of stress-response cascade(s). Understanding the relationships and roles of these genes provides novel insight as to the mechanisms of oxidant toxicity and subsequent adaptive responses. Conversely, Thrb and Gsr were induced exclusively in 2-ppm–exposed animals compared with 5 ppm, suggesting a toxic response specific to the lower dose of O₃.

The role of hormonal factors, particularly thyroid hormone, in O₃ toxicity has been recognized previously (Fairchild and Graham 1963). Recent studies by Huffman et al. (2001) showed that a 2-fold increase in circulating thyroid hormone levels appeared to increase pulmonary toxicity to short-term inhalation to 2 ppm O₃ in rats, suggesting a role for this hormonal reflex. Thyroid hormone has been shown to regulate its own gene expression. Three of the four c-erbA gene products—erbA-α1, erbA-β1, and erbA-β2—encode biologically active thyroid hormone receptors (Teboul and Torresani 1993). Hyperthyroidism in rats produces organ hypertrophy and an increase in circulating levels of IGF and its binding proteins (IGFBP) (Rosato et al. 2002). IGF-1 is the major mediator of growth hormone effects (Iglesias et al. 2001). It has also been observed that expression of IGF and IGFBP may mediate the number and density of thyroid hormone receptors (Pellizas et al. 1998). The 5-fold induction in the expression of thyroid hormone receptor Thrb and 5- to 15-fold suppression in IGF-binding protein are the first observations of O₃-induced alterations in thyroid hormone receptor expression and regulation of Igfbp2. These observations suggest the possible role of Thrb and Igfbp2 in the increased O₃ toxicity observed in hyperthyroid rats (Huffman et al. 2001).

Immediately altered gene expression profiles derived for the rat lung upon exposure to O₃ toxicity in the lung.

Table 5. List of induced (>2-fold) genes that are unique to 2 or 5 ppm O₃.

| Accession no. | Gene symbol | Gene name | Fold change |
|---------------|-------------|-----------|-------------|
| J03933        | Thrb        | thyroid hormone receptor beta, c-erbA-β | 5.32 |
| U73174        | Gsr         | glutathione reductase | 5.21 |
| L08447        | Cxcl2       | CXC chemokine ligand 2 | 4.37 |
| L46791        | Es3         | liver carboxylesterase precursor 10 (carboxylesterase 3) | 3.95 |
| J02650        | Rp19        | ribosomal protein L19 | 3.51 |
| X96394        | Abcc1       | multidrug resistance protein | 2.70 |
| D29796        | Bcar1       | FAK substrate 130 | 2.53 |
| U49082        | Cxcl2       | CXC chemokine ligand 2 | 2.28 |
| D16554        | Ubb         | polyubiquitin | 2.26 |

Table 6. List of suppressed (>2-fold) genes that are unique to 2 or 5 ppm O₃.

| Accession no. | Gene symbol | Gene name | Fold change |
|---------------|-------------|-----------|-------------|
| J07163        | Jun         | c-jun AP1 | 5.26 |
| M84203        | Kcn2        | potassium channel protein (Kshill A) | 5.20 |
| D19862        | Id2         | inhibitor of DNA binding 1 | 4.33 |
| M81855        | Alox1       | multidrug resistance protein 1 | 2.74 |
| D14051        | Nos2        | inducible nitric oxide synthase | 2.61 |
| U45965        | Cxcl2       | CXC chemokine ligand 2 | 2.57 |
| M86389        | Hsp1b       | heat shock 72 kDa protein 1 | 2.55 |
| L29222        | Igf1r       | IGF-1 receptor | 2.50 |
| D16237        | Cxcl2b      | CXC chemokine ligand 2 | 2.48 |

*Genes that were induced and unique to either 2- or 5-ppm–exposed rat lung are listed here. Accession numbers derived from the NCBi Unigene database (http://www.ncbi.nlm.nih.gov/). Gene symbols and names derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). Fold induction in gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).

Table 7. Confirmation of gene array expression by real time RT-PCR for a select list of genes.

| Gene symbol | Gene name          | Gene array | RT-PCR | Gene array | RT-PCR |
|-------------|--------------------|------------|--------|------------|--------|
| c-erbA      | thyroid hormone receptor | 5.0^c       | 3.0    | NC         | NC     |
| c-jun      | transcription factor AP1 | NC         | 5.0    | 3.0        | 1.8    |
| Nos2       | inducible nitric oxide synthase | NC         | 2.0    | NC         | NC     |
| Gsr        | glutathione reductase | 5.0         | 5.2    | NC         | NC     |
| Igfbp2     | insulin-like growth factor binding protein 2 | 0.5 – 15   | 20.0   | 5.0 – 5.5  | 5.5    |

*Genes that are found suppressed/down-regulated and unique to either 2- or 5-ppm–exposed rat lung are listed here. Accession numbers derived from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/). Gene symbols and names derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). Fold induction in gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).

Table 8. Expression of thyroid hormone receptors (Thrbs). Accession numbers derived from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/). Gene symbols and names derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). Fold induction in gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).

| Gene symbol | Gene name | Fold change |
|-------------|-----------|-------------|
| J03933      | Thrb      | 5.32        |
| U73174      | Gsr       | 5.21        |
| L08447      | Cxcl2     | 4.37        |
| L46791      | Es3       | 3.95        |
| J02650      | Rp19      | 3.51        |
| X96394      | Abcc1     | 2.70        |
| D29796      | Bcar1     | 2.53        |
| U49082      | Cxcl2     | 2.28        |
| D16554      | Ubb       | 2.26        |

*Genes that are found suppressed/down-regulated and unique to either 2- or 5-ppm–exposed rat lung are listed here. Accession numbers derived from the NCBi Unigene database (http://www.ncbi.nlm.nih.gov/). Gene symbols and names derived from the Duke Integrated Genomics Database (https://dig.cgt.duke.edu/try_query.php). Fold induction in gene expression. Fold changes in expression of these genes were statistically significant by one-way ANOVA (p < 0.05).
toxic doses of O3 indicated altered expression of an array of genes common to both the concentrations studied (2 and 5 ppm), whereas some were unique to each dose. These profiles represent a spectrum of initiating events and recovery responses. The induced gene involved fatty acid metabolism, cell proliferation, and stress response, and the suppressed genes involved signal mediators, second messenger systems, and G-protein–coupled receptors. The observation of differential expression of Ighbp-2 and Thro8 provides the first biochemical clue for their involvement in O3 toxicity and its exacerbation in hyperthyroid conditions. Increased expression of genes involved in cell proliferation, DNA damage repair, and the stress response, such as Nos2, Gsr, and transcription factors c-jun and NF-κB, suggests the initiation of injury recovery response pathways. Further detailed analysis of these genes and their downstream signaling pathways may shed light on their roles, and they may serve as potential biomarkers for monitoring O3 toxicity. The gene expression profiles presented here were derived from total lung tissue, which could have in part masked or diluted the injury response in airway epithelium. Alternatively, marginated or infiltrating inflammatory cells could have also contributed the gene expression profiles as observed. Gene expression profiles obtained from in vitro studies using airway and bronchial epithelial cells and from BALF cells might expand our understanding of cell specificity in O3 pulmonary toxicity, although the interactions of the various cell types might be lost.

The gene expression profiles derived in the present study provide insights into potential markers of the early O3 response. These markers must now to be evaluated at lower levels of O3 to establish a context within a dose–response model. The goal will be to use these profile maps to relate to mechanisms in human exposure scenarios.

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