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Modulation of ozone-sensitive genes in alpha-tocopherol transfer protein null mice

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Abstract

Alpha-tocopherol transfer protein (ATTP) null mice (ATTP^{-/-}) have a systemic alpha-tocopherol (AT) deficiency, with their lung AT levels being < 10% of those in AT-replete ATTP^{+/+} mice when fed a standard rodent chow diet. ATTP^{+/+} and ATTP^{-/-} mice (4 wk old male mice, *n* = 16 per group) were fed a standard diet (35 IU AT/kg diet) for 8–12 wk, exposed 6 h/day for 3 days to either to O₃ (0.5 ppm) or filtered air, then sacrificed. No significant differences in plasma or lung AT concentrations were observed in response to this level of O₃ exposure. Lung genomic responses of the lungs to O₃ were determined using Affymetrix 430A 2.0 arrays containing over 22,600 probe sets representing 14,000 well-characterized mouse genes. As compared with filtered air exposure, O₃ exposure resulted in 99 genes being differentially expressed in ATTP^{-/-} mice, as compared to 52 differentially expressed genes in ATTP^{+/+} mice. The data revealed an O₃-induced upregulation of genes related to cell proliferation/DNA repair and inflammatory-immune responses in both ATTP^{+/+} and ATTP^{-/-} mice, with the expression of 22 genes being common to both, whereas 30 and 77 genes were unique to ATTP^{+/+} and ATTP^{-/-} mice, respectively. The expressions of O₃ sensitive genes—*Timp1*, *Areg*, *Birc5* and *Tnc*—were seen to be further modulated by AT status. The present study reveals AT modulation of adaptive response of lung genome to O₃ exposure.

Keywords

Alpha-tocopherol; alpha-tocopherol transfer protein null mice; gene expression profiling; oxidative stress; ozone; vitamin E

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Introduction

Ozone (O₃), the principal component of photochemical smog and a powerful highly reactive hydrophobic oxidant, forms peroxides, aldehydes, and lipid ozonation products (LOP) as a result of unsaturated fatty acid oxidation in biological systems (Pryor, 1992, 1994; Pryor et al., 1995). Upper respiratory tract lining fluids (RTLFL) come into direct contact with inhaled O₃, absorbing and detoxifying significant portions of inhaled O₃, thereby reducing amounts of the inhaled toxin reaching more vulnerable peripheral gas exchange regions of the lung (Halliwell and Cross, 1994; Langford et al., 1995). Epidemiological studies have provided evidences that O₃ exposures are related to adverse health effects, including exacerbations of respiratory-tract diseases (Tager et al., 2005) and increased mortality (Thurston and Ito, 2001). Human supplementation with antioxidants, such as α -tocopherol (AT), has been reported to ameliorate decrements in O₃-induced lung injury in some (Samet et al., 2001; Sienra-Monge et al., 2004), but not all (Mudway et al., 2006), studies.

α -Tocopherol (AT), a lipid-soluble dietary antioxidant, is the most abundant and biologically active form of vitamin E (VE) (Schneider, 2005). VE is absorbed into the lymphatics from the intestine and initially transported in chylomicrons to the systemic circulation. Following uptake of the circulating VE-containing chylomicrons and their remnants by the liver, AT is preferentially bound to the hepatic AT transfer protein (ATTP), incorporated into lipoproteins, and secreted into the circulation (Traber and Arai, 1999). ATTP mRNA has been detected in low levels in other tissues, including rat brain, spleen, lung, and kidney (Hosomi et al., 1998). ATTP^{-/-} mice, therefore, have low plasma and extrahepatic tissue AT concentrations (approximately 2–20% of littermate ATTP^{+/+} mice), while liver concentrations remain approximately 40% of those of their ATTP^{+/+} littermates (Leonard et al., 2002).

Previously, we reported genome-wide changes in mRNAs from livers and brain cortex of ATTP^{-/-} mice using high-density oligonucleotide arrays. Our findings included enhanced gene expression related to cell proliferation pathways and oxidant stress in liver and repression of specific genes that determine synaptic plasticity and neuronal development in brain cortex (Gohil et al., 2003b). In a later report our results also suggested age-related multi-organ deregulation of gene networks in ATTP^{-/-} as compared with ATTP^{+/+} mice (Gohil et al., 2004). We have also reported modest increases in lung and liver heme oxygenase 1 (*HO-1*) and inducible nitric oxide synthase (*iNOS*) expression in lipopolysaccharide (LPS)-injected (ip) ATTP^{-/-} mice as compared with ATTP^{+/+} mice (Schock et al., 2004). Recently we reported dysregulated expression of immune- and lipid metabolism-related genes in ATTP^{-/-} heart tissue and dysregulated airway allergic inflammatory responses in ovalbumin-sensitized ATTP^{-/-} mice (Lim et al., 2008; Vasu et al., 2007).

O₃ exposure has been used to explore transcriptomic responses of the lungs to oxidative stress. When C57BL/6J mice were exposed to O₃ (1 ppm for 3 consecutive nights, 8 h/night) followed by microarray analysis of lung mRNA, we observed induction of the expression of serum amyloid A3 gene and of DNA- and cell cycle-related genes, as well as repression of xenobiotic and cytoskeletal genes, as compared with lungs from air-breathing mice (Gohil et al., 2003a). These findings suggest that various defensive mechanisms are activated to protect lungs from O₃ exposure in mice.

AT deficiency to the extent obtained by ATTP^{-/-} mice would be uncommon in humans except for those with severe fat malabsorption or rare families exhibiting ATTP deficiency (Alex et al., 2000; Jeppesen et al., 2000). However, by manipulating systemic AT, a major lipophilic antioxidant, we would predict a robust “antioxidant action,” which would be further augmented by O₃ (Traber and Atkinson, 2007). On the other hand, AT may have biological activities beyond its antioxidant action (Azzi, 2007; Brigelius-Flohe and Davies, 2007). ATTP^{-/-} mice

represent a unique strain where we can study the effect of AT on biological systems by genetically (via deletion of ATTP gene) manipulating systemic AT levels without any dietary AT regulations. The present study was carried out to analyze the genome-wide effect of O₃ in AT-deficient lungs secondary to ATTP deletion in ATTP^{-/-} mice and AT-sufficient mice (ATTP^{+/+}) as compared to their air-breathing ATTP^{-/-} and ATTP^{+/+} littermate controls. Some of the results in this article have been previously reported in abstract form (Vasu et al., 2006a, 2006b).

Materials and methods

Animals, diet, and experimental design

The protocols for the care and use of animals were approved by the Institutional Care and Use Committee at the University of California, Davis. Male C57BL/6 mice with a deletion of ATTP gene (ATTP^{-/-}) and wild-type mice (ATTP^{+/+}) were used from our colonies. The ATTP^{-/-} mice in C57BL6/J genetic background were obtained by backcrossing the original colony (Terasawa et al., 2000) of mixed (50% C57BL6/J and 50% 129/SvJae) mice heterozygous for the deletion (ATTP^{+/-}) with C57BL6/J (ATTP^{+/+}) mice. The mice were housed in polycarbonate cages in a room maintained at 21–23°C and 60–70% humidity on a 12-h light/dark schedule with ad libitum access to water and food. ATTP^{-/-} mice were bred by mating heterozygous (ATTP^{+/-}) male and female mice, and the offspring were genotyped using specific primers for ATTP and confirmed by Western blot analysis using antibody against ATTP protein in liver (Terasawa et al., 2000). After weaning, the offspring were fed diets containing 35 IU *dl*-tocopheryl acetate/kg diet (USB Corporation, Cleveland, OH).

O₃ Exposure

At 12–14 wk, ATTP^{+/+} or ATTP^{-/-} mice were exposed either to filtered air or to 0.5 ppm O₃ for 6 h/day for 3 consecutive days ($n = 8$ per group), with body weights being measured before and after exposure. The O₃ was produced from O₂ by electrical corona arc discharge (sander Ozonizer model IV, Eltze, Germany). The O₂–O₃ mixture (~95% O₂, 5% O₃) was mixed with ambient air and allowed to flow into a Teflon-lined exposure chamber, with the O₃ concentration in chamber being adjusted to 0.5 ppm and continuously monitored by an O₃ detector (Dasibi model 1003-AH, Glendale, CA) (Valacchi et al., 2004). Exposure to filtered air was done in similar exposure chambers except that filtered airflow was released into the chamber at flow rates similar to the O₃ flow. Temperature and humidity were monitored during exposures (25–28°C and 45–55%, respectively).

All the animals were sacrificed immediately after the third 6-h exposure by intraperitoneal injection of beuthanasia (120 mg/kg body weight) and bronchoalveolar lavage (BAL) performed in half of the animals ($n = 4$ per group). Blood was collected by cardiac puncture and lung (dissected free of trachea, large airways, and blood vessels), and liver tissues were rapidly obtained and stored at –80°C until further processing.

AT analyses

Plasma, liver, and lung AT analyses were determined using high-pressure liquid chromatography (HPLC) with electrochemical detection (Podda et al., 1996).

Nitrate/nitrite measurements

Plasma nitrate/nitrite were measured as an indicator of NO production through reduction with acidified vanadium(III) using the Sievers NO analyzer (Sievers, Boulder, CO) (Van Der Vliet et al., 2000).

Bronchoalveolar lavage

Animals were sacrificed, tracheal cannula inserted, and bronchoalveolar lavage (BAL) performed three times via the trachea using approximately 1.0 ml of ice-cold phosphate-buffered saline (PBS, pH 7.4) and recovered by gentle manual aspiration. The recovered BAL fluid was centrifuged, the cell pellet washed twice, and finally resuspended in 1 ml PBS. The total cell count was performed in a Burkert chamber (Novolab, Geraardsbergen, Belgium).

To assess alveolar-capillary membrane protein permeability, total protein from cell-free BAL fluid was measured using a commercially available protein bioassay kit (Bio-Rad Laboratories, Hercules, CA).

RNA extraction and biotin-labeled RNA for gene chip analysis

Total RNA from ~30 mg unlavaged lung tissue was extracted by RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA purity and quantity were determined spectrophotometrically (A_{260}/A_{280} ratio being close to 2.0 for pure RNA). Four replicates, each from a mouse lung, from each group ($n = 4$ per group), were further processed as follows for gene chip analysis.

The methods used to prepare the eukaryotic target preparation were followed according to the manufacturer's protocol (Affymetrix, Inc.) for expression arrays. An aliquot (10 μ g) of RNA solution was used for preparation of one-cycle cDNA synthesis (first-strand and second-strand cDNA synthesis) followed by cleanup of double-stranded cDNA and synthesis of biotin-labeled cRNA. The biotin-labeled cRNA (40 μ g) was used for fragmenting for target preparation. Fragmented cRNA samples were hybridized to high density oligonucleotide Affymetrix Mouse 430A 2.0 GeneChips (Santa Clara, CA) which contains 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes. For hybridization, gene expression was assessed using one chip per mouse lung.

Oligonucleotide microarray analysis and statistics

The scanned images of hybridization signals were analyzed with the Affymetrix GeneChip Operating Software (GCOS) including the GeneChip Scanner 3000 High-Resolution Scanning Patch and DNA-D chip analyzer (dChip), a software package implementing model-based expression analysis of oligonucleotide arrays at <http://www.dchip.org> (Li and Hung Wong, 2001). The absolute mRNA expression (present or absent) and differential mRNA expression data were obtained from GCOS. When the p value for detection signal was $< .049$ (range of p value .0002–.049), the expression of the mRNA was classified as present (P). All mRNAs with the p value for detection $> .05$ were considered absent (A). The signal intensities for transcripts classified as present ranged from 5 to 7000 U.

The entire data of ATTP^{+/+}, ATTP^{-/-}, air/O₃-exposed groups of mice were subjected to analysis. CEL files generated in GCOS were analyzed using dChip (version 1.0) by model-based expression (perfect match/mismatch; PM/MM) analysis to compute expression values. Hybridization data from O₃-exposed lungs were treated as “Test” data and those for control, air-exposed lungs as “Baseline” data. The generated gene list was filtered representing the following criteria: (i) data that showed either an “increase” or a “decrease” of ≥ 2 -fold expression comparing air-exposed ATTP^{-/-} group of mice versus ATTP^{+/+} groups; (ii) O₃-exposed ATTP^{+/+}/ATTP^{-/-} groups of mice versus air-exposed ATTP^{+/+}/ATTP^{-/-} groups greater than 100 expression (E) value difference in either direction (i.e., $E_{\text{test}} - E_{\text{control}} > 100$ or $E_{\text{control}} - E_{\text{test}} > 100$); (iii) p for testing was set at $< .05$, where p is based on the t distribution used to assess the relative magnitude of the expression value difference. The D-Chip software program calculates mean and standard error of the mean (SEM) for each mRNA in a baseline (e.g., air) sample and compares the mean and SEM for the same mRNA in test sample (e.g.,

O₃). The program then gives a *p* value for the difference and magnitude of the difference. The data are then edited to select only the genes that show ≥ 2 -fold and *p* value $< .05$. Our previous experience (Gohil et al., 2003a, 2003b, 2007; Oommen et al., 2007; Vasu et al., 2007, 2009) has shown that we can confirm “all” the changes that are ≥ 2 -fold, by independent quantitative real-time polymerase chain reaction (PCR) assay using a sample size of 4–6 mice per group. However, the entire data from this study will be submitted online at Array Express (<http://www.ebi.ac.uk/arrayexpress>). Hierarchical clustering or “heat map” of the resulting differentially regulated gene list was analyzed by dChip software.

Validation of GeneChip data by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from lung as described for GeneChip analysis and cDNA was synthesized from 5 μ g total RNA and reverse transcribed to obtain cDNA. Gene-specific primers (Table 1) for selected genes were designed using Primer Express 2.0 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used to normalize for gene expression level. qRT-PCR was carried out with SYBR Green I Master Mix (Applied Biosystems) reagent. The PCR results were analyzed using the ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calculate relative changes in gene expression determined from qRT-PCR experiments (Applied Biosystems User Bulletin No. 2 [P/N4303859]). The threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the SYBR Green emission increases above a preset threshold level. The specific mRNA transcripts were expressed as fold difference in the expression of the specific mRNAs in RNA samples from the lungs of ATTP^{+/+}/ATTP^{-/-} mice exposed to O₃ compared to those in the respective lungs of mice exposed to filtered air.

Statistical analyses—Statistical evaluation of analytical data was done by Student's *t* test using the statistical software GraphPad Prism 4.0 (San Diego, CA). In all comparisons *p* $< .05$ was considered as significant. Results are expressed as mean $\pm$ SEM. Tissue AT concentrations were evaluated by ANOVA after log transformation of data, and Tukey's multiple comparison test was employed for pairwise comparisons.

Results

Plasma, liver or lung AT levels

The plasma, liver, or lung AT levels in ATTP^{-/-} mice were 10-to 20-fold lower than those of ATTP^{+/+} mice (Figures 1–3). Though O₃ exposure (0.5 ppm, 6 h/day for 3 days) caused a significant decrease (*p* $< .01$) in liver AT concentrations of ATTP^{-/-} mice as compared to air-exposed ATTP^{-/-} mice, no significant changes were observed in liver AT levels of ATTP^{+/+} mice (Figure 2). Plasma and lung AT levels were unaffected by O₃ exposure in both genotypes (Figures 1 and 3, respectively).

Plasma nitrate/nitrite levels

No significant changes in plasma nitrate/nitrite levels were observed in ATTP^{+/+}/ATTP^{-/-} mice exposed to O₃ as compared to their respective air-breathing controls (data not shown).

Total BAL cell count and protein levels

Significantly (*p* $< .001$) increased levels of total BAL cell counts and cell-free BAL protein levels were observed in response to O₃ exposure in both groups of mice (Figures 4 and 5). Notably, the total BAL cell count doubled in response to O₃ exposure in ATTP^{-/-} (*p* $< .001$) as compared with ATTP^{+/+} mice (Figure 4).

Microarray analysis

The global gene expression profile of unlavaged lung tissue of $ATTP^{+/+}/ATTP^{-/-}$ mice exposed to O_3 (0.5 ppm, 6 h/day for 3 days) were analyzed and compared to their air-breathing littermate $ATTP^{+/+}/ATTP^{-/-}$ controls. The microarray analysis by mouse 430 A2.0 arrays or gene chips (4 replicates per group) detected ~15,000 probe sets (~9000 genes) in both O_3 -exposed $ATTP^{+/+}$ and $ATTP^{-/-}$ lung tissues. O_3 exposure resulted in the selective modulation of 52 and 99 genes in the lungs of $ATTP^{+/+}$ and $ATTP^{-/-}$ mice, respectively. The expression of 22 genes was common to both (Table 2), of which the expressions of 4 genes (*Timp1*, *Areg*, *Birc5*, *Tnc*) were seen to be further modulated by lung AT status. The expression of 30 and 77 genes were unique to $ATTP^{+/+}$ and $ATTP^{-/-}$ mice, respectively (Figure 6). Figure 7 shows the “heat map” for the entire set of differentially regulated genes. The coefficient of variation for Chip–Chip, i.e., same sample on four different chips, was <2%. As reported earlier by us (Oommen et al., 2007), mouse-to-mouse variation depends on the activity of the gene and can vary from 5 to 41%. Our selection criterion for a significant change was empirically set at $\geq 100\%$.

Comparing lung mRNA expression in air-breathing mice, only two genes, *Prrx1* and *Hspb1*, were downregulated, ~4- and 3-fold, respectively, in $ATTP^{-/-}$ compared with $ATTP^{+/+}$ mice (Table 3). It must be noted that as compared to our earlier published reports (Oommen et al., 2007; Vasu et al., 2007), in this study we have used more stringent criteria for selection of genes that are sensitive to AT deficiency and/or O_3 . The primary aim was to obtain the most sensitive genes that have high statistical probability to be “real change” and possibly have biological significance. However, the entire data set from this study will be published online at Array Express (<http://www.ebi.ac.uk/arrayexpress>).

Table 4A shows functional groups of selected genes that were upregulated in $ATTP^{+/+}$ mice in response to O_3 exposure. Most of these genes were related to inflammatory-immune responses and cell proliferation or DNA repair. Some of these included FK506 binding protein 5 (*Fkbp5*), metallothionein 2 (*Mt2*), chemokine (C-X-C motif) ligand 2 (*Cxcl2*), prostaglandin-endoperoxide synthase 1 (*Ptgs1*), cell division cycle 2 homolog A (*Cdc2a*), and cell division cycle 6 homolog (*Cdc6*). The expression of transcription factors such as period homolog 1 (*Per1*), nuclear factor interleukin 3 regulated (*Nfil3*), and activating transcription factor 3 (*Atf3*) was also seen to be upregulated. Interestingly, only seven genes were found to be downregulated in $ATTP^{+/+}$ mice in response to O_3 exposure as compared to their air-breathing controls (Table 4B). These included immunoglobulin heavy chain (*Igh-VJ558*), N-acylsphingosine amidohydrolase 3-like (*Asah3l*), hepatitis A virus cellular receptor 1 (*Havcr1*), casein kappa (*Csnk*), membrane-spanning 4-domains, subfamily A, member 4C (*Ms4a4c*), WNT1 inducible signaling pathway protein 2 (*Wisp2*), and protocadherin 18 (*Pcdh18*), of which *Igh-VJ558* showed a 36-fold downregulation. The entire list of differentially expressed genes is shown in Appendix I.

The lung gene expression of $ATTP^{-/-}$ mice when exposed to O_3 showed 99 genes differentially expressed as compared to their air-breathing $ATTP^{-/-}$ controls. This was approximately 50% more than that observed in differential comparison of $ATTP^{+/+}$ lungs exposed to O_3 and respective air-breathing controls (Figure 6). The functional classification of these upregulated genes resulted in six groups: cell proliferation- or DNA repair-related genes (40%), inflammatory-immune related genes (27%), transcription-related genes (7%), cytoskeleton-related genes (3%), lipid metabolism-related genes (2%), and ion transport-related genes (1%) (Table 5A). Seven genes were found to be downregulated in lungs of $ATTP^{-/-}$ mice exposed to O_3 as compared to their air-breathing controls (Table 5B). These included membrane-spanning 4-domains, subfamily A, member 4c (*Ms4a4c*) (–3.5-fold), complement receptor 2 (*Cr2*) (–3.3-fold), interferon-induced transmembrane protein 6 (*Ifitm6*) (–3.3-fold), CD19 antigen (*Cd19*) (–3.1-fold), ATPase, Na^+/K^+ -transporting, alpha 3 polypeptide (*Atp1a3*) (–3.0-

fold), nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*) (−3.0-fold), and insulin-like growth factor binding protein 3 (*Igfbp3*) (−2.9-fold). The entire list of differentially expressed genes is shown in Appendix II.

Validation of microarray data

We selected 14 genes from the microarray data to confirm independently by qRT-PCR. The selected genes included upregulated (*Gclc*, *Saa3*, *Marco*, *Timp1*, *Areg*, *Check1*, *Cxcl2*, *Fkbp5*, *Tnc*, *Ccna2*, *Mbd1*) and downregulated (*Nr1d1*, *Hspb1*, *Igfbp3*) genes. The fold-change data analyzed by qRT-PCR were similar to or lower than the microarray data and was highly correlated ($R^2 = .8076$) (Figure 8). The higher degree of consistency between the qRT-PCR data and oligonucleotide microarray data raised confidence in the gene expression data obtained and the validity of the experiments.

Discussion

ATTP^{−/−} mice represent a unique strain in which one can study the effect of AT on biological systems by genetically (via deletion of ATTP gene) manipulating systemic AT levels. The experimental design utilized ATTP^{−/−} mice in which extremely low levels of lung AT were achieved secondary to ATTP deletion. The present study was carried out to explore the potential of the major nutritional lipophilic antioxidant, AT, on the adaptive response of lung transcriptomes to O₃, the most important photochemical oxidant. Genome-wide effect of O₃ were analyzed in ATTP^{−/−} mice and AT-sufficient mice (ATTP^{+/+}) as compared to their respective air-exposed ATTP^{−/−} and ATTP^{+/+} littermate controls.

The AT levels in plasma, liver, and lung tissues of ATTP^{−/−} mice were much lower as compared to their respective levels in ATTP^{+/+} littermate controls, as reported earlier by us and by other workers (Gohil et al., 2003b, 2004; Leonard et al., 2002; Lim et al., 2008; Schock et al., 2004; Terasawa et al., 2000). We observed an increase in total BAL cell count and protein levels in O₃-exposed mice, confirming O₃-induced inductions of lung injury (Guth et al., 1986), but we did not find any significant change in plasma or lung AT levels in O₃-exposed ATTP^{+/+} or ATTP^{−/−} mice (0.5 ppm, 6 h/day for 3 days) as compared to their respective air-exposed littermate controls. However, a significant O₃-induced drop was observed in liver AT levels in ATTP^{−/−} mice as compared to air-exposed ATTP^{−/−} mice (Figure 2). A possible explanation would be an increase in circulatory lipid hydroperoxides as a result of AT deficiency in O₃-exposed ATTP^{−/−} mice as observed by other workers in O₃-exposed AT deficient rats (Sato et al., 1980). In a study published in 1980, rats fed vitamin E-deficient diet or vitamin E-supplemented diet were exposed to 0.3 ppm O₃ for 3 h daily, 5 days/wk, for 7 mo (Sato et al., 1980). The investigators observed that serum vitamin E concentration decreased following O₃ exposure in the E-supplemented group, but remained unaffected in the E-depleted group. Interestingly, they observed that liver tissues showed more damaging affect as compared to lung tissues that were directly exposed to O₃, as reflected by biochemical and morphological observations, although no clear explanation could be put forth (Sato et al., 1980). In another study, in a burn and inhalation injury model in sheep, Traber and workers observed nearly 2000 nmol alpha-tocopherol depletion from the liver, as compared to only 1000 nmol alpha-tocopherol loss in lungs, over 48 h after exposure to burn and inhalation injury (Traber et al., 2007). The loss of hepatic alpha-tocopherol was suggested to be caused by lipid hydroperoxides, which are delivered to the liver by lipoproteins (Traber et al., 2007).

We had earlier reported a decrease in plasma and lung AT levels (23 and 20%, respectively) in O₃-exposed SKH-1 hairless mice (0.8 ppm, 6 h/day for 6 days) as compared to their air-breathing controls (Valacchi et al., 2004). Elsayed and workers have previously reported an increase in lung AT content after O₃ exposure (0.5 ppm, 5 days) in Long-Evans rats fed an AT-supplemented diet, suggesting mobilization of AT to lungs as a response to “oxidative

stress” or as a result of O₃-induced inflammatory responses with leakage of blood constituents into lung tissues (Elsayed et al., 1990). In another study, no significant changes were found in plasma AT levels in mature female rabbits exposed to 0.1 to 0.6 ppm of O₃ for 3 h (Canada et al., 1987). These observations suggest the degree of oxidative stress caused by O₃ exposure, potentially dependent upon the duration of exposure, the levels of antioxidant (both enzymatic and nonenzymatic), the magnitude of the inflammatory responses, and/or the species/strain of animals may all be involved in modulating lung AT levels.

Comparing the differential expression of lung genes in air-breathing mice, only two genes, paired related homeobox 1 (*Prrx1*) and heat-shock protein 1 (*Hspb1*), were downregulated, by 4.2- and 3.2-fold, in ATTP^{-/-} lungs (Table 3). *Prrx1* (also known as *Prx1* or *Mhox*) is a transcription factor associated with differentiation and vascularization of vessel walls (Bergwerff et al., 1998). In lungs, *Prrx1* has been localized to the differentiating endothelial cells and *Prrx1*^{-/-} mice have been reported to show abnormal lung vascularization (Ihida-Stansbury et al., 2004). *Hspb1* (also known as *Hsp27* or *Hsp25*) belongs to the family of small heat-shock proteins that are stimulated by heat shock or disturbed cell environment or inflammation. *Hspb1* was also suggested to be anti-apoptotic effector acting by sequestering cytochrome *c* released from mitochondria (Bruey et al., 2000). Sinha et al. reported that AT deficiency leads to reduced *Hspb1* expression in lung type II pneumocytes and thus promotes apoptosis (Sinha et al., 2002). Reduced *Hspb1* expression was assumed to be dependent upon the protein kinase C (PKC) pathway, as AT is known to modulate PKC activity (Mahoney and Azzi, 1988). In type II pneumocytes its deficiency has been reported to strongly increase PKC activity (Sabat et al., 2001). The present study further supports a role for AT in *Hspb1* expression.

Of note, we were surprised to observe that only two genes were differentially expressed in ATTP^{-/-} lungs as compared to lungs of respective littermate ATTP^{+/+} controls. We were expecting a “significant number” of differentially expressed genes, particularly those related to “oxidant responsive” genes. Interestingly, we previously have not observed any significant increase in isoprostanes in lungs from ATTP^{-/-} mice (unpublished data, from the laboratory of the late Dr. J. Morrow) as compared to ATTP^{+/+} controls. Similar unexpected observations were recently reported by other workers in brain tissue of ATTP^{-/-} mice (Cuddihy et al., 2008).

O₃-sensitive lung genes modulated by AT status

Of the expression of 22 genes in lungs common to both ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃, the expression of 4 genes [tissue inhibitor of metalloproteinase 1 (*Timp1*), amphiregulin (*Areg*), baculoviral IAP-repeat containing 5 (*Birc5*), and Tenascin C (*Tnc*)] was seen to be further modulated by lung AT status (Table 2). *Timp1*, *Areg*, *Birc5*, and *Tnc* were induced 4.1-, 5.9-, 4.7-, and 4.4-fold in ATTP^{+/+} mice, whereas they were induced by 13.8-, 8.2-, 8-, and 8-fold, respectively, in ATTP^{-/-} mice. TIMPs are regulators of matrix metalloproteinase activity and their levels are known to be increased in lung injury (Kim et al., 2005). They have also been touted as a physiological regulator of normal airway repair (Chen et al., 2008). Wang et al. have reported a consistent increase in *Timp1* mRNA expression in rat type I-like lung pneumocytes exposed to 100 ppb (0.1 ppm) O₃ for 1 h, thus suggesting the importance of this lung cell type in alveolar injury repair (Wang et al., 2006). Similarly, the expression of amphiregulin (*Areg*) was induced 8.2-fold in O₃-exposed ATTP^{-/-} mice, as compared to 5.9-fold in O₃-exposed ATTP^{+/+} mice. *Areg*, a member of epidermal growth factor, is known to regulate cell proliferation (Schuger et al., 1996). Shao and workers have reported that *Areg* can be activated by prostaglandin E(2) (PGE₂), a major product of cyclooxygenase (*cox*) via cAMP/protein kinase A pathways in colon cancer cells (Shao et al., 2003).

Of note, in the present study, the mRNA expression of prostaglandin-endoperoxide synthase 1 and 2 (*Ptgs1* and 2/*Cox-1* and 2) was seen to be induced by 2.5- and 3.8-fold, respectively, in O₃-exposed ATTP^{-/-} mice. Interestingly, only *Ptgs1* was seen to be induced (2.4-fold) in O₃-exposed ATTP^{+/+} mice. AT supplementation has been reported to reverse age-associated increase in murine macrophage PGE₂ production and COX activity, but had no effect on COX 1 and 2 protein expression or COX 2 mRNA expression (Wu et al., 1998). The authors suggested that AT may be exerting its effect posttranslationally by inhibiting COX activity (Wu et al., 1998). Hence our microarray data suggest that O₃ exposure to lung tissues with low AT levels (ATTP^{-/-} mice) may increase *Ptgs1* and -2 expressions, leading to increased expression of *Areg*, which in turn may modulate cell proliferation mechanisms. Similarly, the increased expression of tenascin C (*Tnc*), an extracellular matrix component, in lung tissues of O₃-exposed ATTP^{-/-} mice (8-fold) as compared to lung tissues of O₃-exposed ATTP^{+/+} mice (4.4-fold) further consolidates our hypothesis of modulated lung injury pathways in O₃-exposed ATTP^{-/-} mice as a result of low AT status. *Tnc* is associated with cell detachment, migration, proliferation, and tissue remodeling (Chiquet-Ehrismann, 1991). Potter-Perigo and workers have shown an increased expression of *Tnc* in O₃-exposed (0.3 ppm, 3 h) primate nasal epithelial cells (harvested after 24 h postexposure) as a result of epithelial cell detachment and disruption (Potter-Perigo et al., 1998).

The increased expression of *Birc5* (also known as survivin), a member of the inhibitor of apoptosis protein family (IAP) (Salvesen and Duckett, 2002), in lungs of O₃-exposed ATTP^{-/-} mice (8-fold) represents another marker of inflammation (Altnauer et al., 2004). Overexpression of *Birc5* had also been reported in proliferating cells and its expression had been described to be cell-cycle dependent (Altnauer et al., 2004). Thus the present data reveal O₃-sensitive genes that may be further modulated by AT levels in lung tissues. It must also be noted that the absence of any dose response or time course of O₃ exposure in the present study (one of the limitation of the study) renders it difficult to discern direct O₃-specific changes from those of O₃ plus the resulting inflammatory-immune system activation-induced changes. Similarly, another limitation of the current study includes the difficulty in ascribing noted changes to changes in a specific lung cell population. However, it should also be recognized that the GeneChip assay used in this study is very sensitive with a large dynamic range for detections (signal intensity of 10–10,000 units) (Oommen et al., 2007). Hence by pairwise comparison of two treatment groups, some of the large changes in even a small population of lung cells should be detected.

Differential expression of genes upregulated in O₃-exposed lungs of ATTP^{+/+} and ATTP^{-/-} mice

Comparative analysis of lung gene expression in ATTP^{-/-} mice exposed to O₃ as compared to their respective air-breathing ATTP^{-/-} control mice showed similar trends in upregulation of inflammatory-immune related and cell differentiation/DNA repair/DNA synthesis related genes as observed in lungs of ATTP^{+/+} mice exposed to O₃. The changes in gene expression in lungs of ATTP^{-/-} mice in response to O₃ were significantly higher, as seen by the differential expressions of greater than 50%, likely due to AT deficiency as a result of ATTP gene deletion (Figure 6).

We have earlier reported an induction of lung mRNA expression of serum amyloid A3 (*Saa3*), a main acute-phase protein in mice exposed to 1 ppm O₃ for 3 consecutive nights (8 h/night) (Gohil et al., 2003a). In the current study we did not see any change in *Saa3* expression in ATTP^{+/+} mice exposed to O₃. However, there was an 18.7-fold increase in *Saa3* expression in ATTP^{-/-} mice exposed to O₃ (Table 5A). *Saa3* has been shown to induce extracellular matrix-degrading enzymes such as collagenase and matrix metalloproteinases (Mitchell et al., 1991; Vallon et al., 2001). The expression of macrophage receptor with collagenous structure

(*Marco*), class A scavenger receptor, was upregulated by 6.1-fold in lungs of O₃-exposed ATTP^{-/-} mice. Its expression was reported to be restricted to subsets of macrophages active in scavenging pathogenic microorganisms (Kraal et al., 2000). Dahl and workers recently reported increased mRNA expression of *Marco* in lungs in response to O₃ exposure in wild-type mice and at the same time reported increased lung inflammation and injury in MARCO^{-/-} mice instilled (intratracheally) by pro-inflammatory O₃-generated lipids: 5 β ,6 β -epoxycholesterol (β -epoxide) and 1-palmitoyl-2-(9'-oxo-nonanoyl)-glycerophosphocholine (PON-GPC) (Dahl et al., 2007). The unavailability of time-course observation in the present study makes it difficult to explain the modest increase (6.1-fold in lungs of O₃-exposed ATTP^{-/-} mice) in MARCO mRNA expression as compared to reports by Dahl and workers (Dahl et al., 2007) in a different cohort of mice.

Several genes related to cell proliferation markers and regulators, such as cyclin A2 (*Ccna2*), cyclin B1 (*Ccnb1*), Cyclin B2 (*Ccnb2*), *Chek1*, *Tnfrsf12a*, *Gadd45g*, and *Top2a*, were also upregulated in O₃-exposed ATTP^{-/-} mice. Alpha-fetoprotein (*Afp*), reported to promote cell proliferation of NIH 3T3 cells by induction of the PKA-cAMP pathway (Li et al., 2002), was also upregulated.

As compared to respective air-breathing controls, in lungs of ATTP^{+/+} mice exposed to O₃, chemokine (C-X-C motif) ligand 2 (*Cxcl2*), also known as macrophage inflammatory protein 2 (*Mip-2*), was induced by 5-fold. *Cxcl2* has been reported to be secreted from alveolar macrophages, neutrophils, type II epithelial cells, and lung fibroblasts (Driscoll et al., 1993a) and is believed to play an important role in O₃-induced inflammatory responses (Driscoll et al., 1993b). Tumor necrosis factor receptor superfamily, member 12a (*Tnfrsf12a*) (also known as *TWEAK-R* or *Fn14*), predominantly found in macrophages and a well-known modulator of cell proliferation, differentiation, and apoptosis (Girgenrath et al., 2006), was induced by 2.8-fold. Similarly, cell cycle-related genes such as cell division cycle 2 homolog (*Cdc2*), cyclin A2 (*Ccna2*), survivin (baculoviral IAP repeat-containing 5, *Birc5*), mitotic arrest deficient, homolog (*MAD2*), cell cycle division 20 homolog (*Cdc20*), cell division cycle 6 homolog (*Cdc6*), topoisomerase DNA II alpha (*Top2A*), and other related genes were also induced.

Dysregulated expression of “clock genes” in O₃-exposed lungs of ATTP^{+/+} and ATTP^{-/-} mice

Transcription factors such as period homolog 1 (*Per1*), nuclear factor, interleukin 3 regulated (*Nfil3*), and activating transcription factor 3 (*Atf3*) were upregulated by 3.2-, 3.2-, and 2.9-fold, respectively, in lungs of O₃-exposed ATTP^{+/+} mice (Table 4A). *Per1*, implicated in regulating circadian rhythm, has been reported to be elevated in the paraventricular nucleus of mice as a result of stress-induced disturbance of circadian corticosterone secretion (Takahashi et al., 2001). The expression of the “clock genes” including *Per1* has been reported to be present in larynx, trachea, bronchus, and lung of mice (Bando et al., 2007) and has also been reported to regulate cell cycle by interacting with components of the cell cycle checkpoint system activating checkpoint kinase 1 and 2 (*Chek1* & 2) in response to DNA damage (Gery et al., 2006). Interestingly, *Chek1* and another key regulator of cell cycle progression, growth arrest and DNA-damage-inducible 45 gamma (*Gadd45g*), were seen to be induced by 4.3- and 3.0-fold in lungs of O₃-exposed ATTP^{+/+} mice. Kondratov and Antoch have reported that *Gadd45g* showed circadian patterns of recognition at mRNA or protein levels (Kondratov and Antoch, 2007). *Nfil3* (also known as *E4BP4*), a mammalian basic leucine zipper (bZIP) transcription factor found primarily in T cells (Cowell, 2002) and induced by interleukin (IL)-3 (Ikushima et al., 1997), has been reported to regulate inflammatory processes and apoptosis and also involves circadian clock mechanisms (Cowell, 2002). *Atf3*, a member of the ATF/CREB family of transcription factors, is a stress-inducible gene induced by a variety of stress signals and also induced by ischemia and hypoxia (Ameri et al., 2007; Chen et al., 1996; Hai et al., 1999). It is also reported to affect cell cycle progression and apoptosis (Lu et al., 2006).

Nr1d1 (Nuclear receptor subfamily 1, group D member 1), also known as Rev-erb alpha, is seen to be downregulated (3-fold) in lungs of O₃-exposed ATTP^{-/-} mice (Table 5B). It is a member of the “orphan” nuclear receptor family (Chawla and Lazar, 1993). *Nr1d1* has also been reported to display circadian rhythmic expression by regulating or being regulated by other “clock genes” of circadian circuitry. It is also known to regulate various metabolic pathways (Duez and Staels, 2008; Torra et al., 2000). Though it is not very clear how O₃ may directly/indirectly influence the expression of genes involved in circadian pathways, studies indicate that oxidative stress might play an important role in their transcriptional regulation (Igarashi et al., 2007).

Summary of limitations and conclusion

One of the limitations of the study was nonavailability of data for O₃-modulated lung gene expression profiles and/or lung injury at different time intervals (van Bree et al., 2001). It must be noted that our transcriptomic data were in lungs exposed to O₃ for 3 days, a period in which the lung epithelial cell proliferation and alveolar wound repair mechanisms and inflammatory responses are simultaneously occurring (Dormans et al., 1999; Savov et al., 2004). The present study did not characterize lipid ozonation products that may have directly or indirectly modulated gene expression as a result of increased lipid peroxidation due to AT deficiency (Kafoury et al., 2007). Other workers have provided evidence that O₃-induced inflammatory responses may be maximally present on the third day of acute O₃ exposure (Dormans et al., 1999; Savov et al., 2004). Bioinformatic analyses of several genes modulated in the present study reveals involvement of nuclear factor (NF)-κB and related pathways (Bing et al., 2000; Cavin et al., 2004; Girgenrath et al., 2006; Gohil et al., 2003a; Huffman et al., 2002; Morante et al., 2005). The induction of the catalytic subunit of glutamate-cysteine ligase, *Gclc*, a rate-limiting enzyme in de novo reduced glutathione (GSH) synthesis, by 2.9-fold in O₃-exposed ATTP^{-/-} mice (Table 5A) also suggests the involvement of a transcription factor of the cap'n'collar-basic leucine zipper proteins (CNC-bZIP), *Nrf2* (Wild et al., 1999). Thus, the present data also suggest possible highly complex cross-talk mechanisms involved between the “stress-responsive” transcription factors such as *Nrf2*, *NF-κB*, and *Atf3*. The present study also identifies four O₃-sensitive lung genes (*Timp1*, *Areg*, *Birc5*, and *Tnc*) that appear to be further modulated by AT status. However, it must also be noted that in the present study we exposed the mice to O₃ (0.5 ppm) 6 h/day for 3 days and thus the gene expression pattern observed represents changes due to O₃, O₃-induced injury-inflammation-repair processes, and/or adaptive responses to O₃ exposure, thus significantly limiting mechanistic interpretations regarding respiratory tissue overall pathobiological responses. However, it can be reasonably concluded that lung genomic responses to O₃ are modulated in ATTP^{-/-} mice compared to their littermate ATTP^{+/+} mice.

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Appendix I

Differentially expressed genes in lungs of ATTP^{+/+} mice exposed to O₃ as compared to their respective air-exposed controls.

Description	Affymetrix probe set ID	Fold change	p Value
FK506 binding protein 5, <i>Fkbp5</i>	1448231_at	10.9	.046047
Amphiregulin, <i>Areg</i>	1421134_at	5.9	.001613
Metallothionein 2, <i>Mt2</i>	1428942_at	5.7	.039341
Cell division cycle 20 homolog (S. cerevisiae), <i>Cdc20</i>	1439394_x_at	5.7	.01147
Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme, <i>Gcnt2</i>	1451733_at	5.4	.006715
Chemokine (C-X-C motif) ligand 2, <i>Cxcl2</i>	1449984_at	5.3	.018505
Cell division cycle associated 1, <i>Cdca1</i>	1430811_a_at	5.1	.016262
C-type lectin domain family 4, member n, <i>Clec4n</i>	1425951_a_at	5.0	.003182
Similar to histone 2a, <i>MGC73635</i>	1438009_at	4.9	.002595
Mitogen-activated protein kinase kinase 6, <i>Map3k6</i>	1449901_a_at	4.8	.004217
Ribonucleotide reductase M2, <i>Rrm2</i>	1416120_at	4.8	.010085
Baculoviral IAP repeat-containing 5, <i>Birc5</i>	1424278_a_at	4.7	.011477
A disintegrin-like and metallopeptidase (repolyisin type) with thrombospondin type 1 motif, 4, <i>Adams4</i>	1452595_at	4.5	.010648
Tenascin C, <i>Tnc</i>	1416342_at	4.4	.005287
Cell division cycle 2 homolog A (S. pombe), <i>Cdc2a</i>	1448314_at	4.4	.001511
Checkpoint kinase 1 homolog (S. pombe), <i>Chek1</i>	1450677_at	4.3	.028655
Tissue inhibitor of metalloproteinase 1, <i>Timp1</i>	1460227_at	4.1	.003086
Similar to thrombospondin 1, <i>LOC640441</i>	1450377_at	4.0	.003209
Lipin 2, <i>Lpin2</i>	1460290_at	4.0	.010188
CEA-related cell adhesion molecule 1, <i>Ceacam1</i>	1452532_x_at	4.0	.026812
Cell division cycle 6 homolog (S. cerevisiae), <i>Cdc6</i>	1417019_a_at	3.8	.019495
Serine (or cysteine) peptidase inhibitor, clade E, member 1, <i>Serpine1</i>	1419149_at	3.5	.009815
Ubiquitin-conjugating enzyme E2C, <i>Ube2c</i>	1452954_at	3.5	.003733
Natriuretic peptide receptor 3, <i>Npr3</i>	1448024_at	3.4	.006901
Iduronate 2-sulfatase, <i>Ids</i>	1450166_at	3.4	.008805
Thrombospondin 1, <i>Thbs1</i>	1460302_at	3.3	.013611
Polo-like kinase 4 (<i>Drosophila</i>), <i>Plk4</i>	1426580_at	3.3	.00878
Rac GTPase-activating protein 1, <i>Racgap1</i>	1451358_a_at	3.2	.010008
Period homolog 1 (<i>Drosophila</i>), <i>Per1</i>	1449851_at	3.2	.002077

Description	Affymetrix probe set ID	Fold change	p Value
Nuclear factor, interleukin 3, regulated, <i>Nfil3</i>	1418932_at	3.2	.002181
Coagulation factor III, <i>F3</i>	1417408_at	3.0	.006349
Growth arrest and DNA-damage-inducible 45 gamma, <i>Gadd45g</i>	1453851_a_at	3.0	.022487
Activating transcription factor 3, <i>Aff3</i>	1449363_at	2.9	.009167
Transmembrane protein 37, <i>Tmem37</i>	1417611_at	2.9	.003164
Tumor necrosis factor receptor superfamily, member 12a, <i>Tnfrsf12a</i>	1418571_at	2.8	.006708
Ribonucleotide reductase M1, <i>Rrm1</i>	1448127_at	2.7	.004612
Basic leucine zipper and W2 domains 1, <i>Bzw1</i>	1450846_at	2.7	.011171
Prostaglandin-endoperoxide synthase 1, <i>Ptgs1</i>	1423414_at	2.4	.002984
Immunoglobulin heavy chain (J558 family), <i>Igh-VJ558</i>	1427839_at	-36.2	.030697
N-Acylsphingosine amidohydrolase 3-like, <i>Asah3l</i>	1421496_at	-6.2	.001439
Hepatitis A virus cellular receptor 1, <i>Havcr1</i>	1450364_a_at	-5.8	.022315
Casein kappa, <i>Csnk</i>	1419735_at	-4.7	.015088
Membrane-spanning 4-domains, subfamily A, member 4C, <i>Ms4a4c</i>	1450291_s_at	-4.4	.017093
WNT1 inducible signaling pathway protein 2, <i>Wisp2</i>	1419015_at	-3.3	.013676
Protocadherin 18, <i>Pcdh18</i>	1422889_at	-2.9	.00596

Appendix II

Differentially expressed genes in lungs of ATTP^{-/-} mice exposed to O₃ as compared to their respective air-exposed group.

Description	Affymetrix Probe set ID	Fold change	p Value
Serum amyloid A 3, <i>Saa3</i>	1450826_a_at	18.7	.008959
Tissue inhibitor of metalloproteinase 1, <i>Timp1</i>	1460227_at	13.8	.001442
Bromodomain and WD repeat domain containing 3, <i>Brwd3</i>	1441182_at	10.1	.004669
Resistin like alpha, <i>Retnla</i>	1449015_at	8.4	.000305
Tenascin C, <i>Tnc</i>	1416342_at	8.0	.000555
Baculoviral IAP repeat-containing 5, <i>Birc5</i>	1424278_a_at	8.0	.001819
Amphiregulin, <i>Areg</i>	1421134_at	8.2	.000441
Cyclin A2, <i>Ccn2</i>	1417910_at	7.2	.000661
Kinesin family member 22, <i>Kif22</i>	1437716_x_at	6.3	.005116
Metallothionein 2, <i>Mt2</i>	1428942_at	6.2	.005926
Macrophage receptor with collagenous structure, <i>Marco</i>	1449498_at	6.1	.006801
Similar to histone 2a, <i>MGC73635</i>	1438009_at	6.1	.02903
Sperm associated antigen 5, <i>Spag5</i>	1433893_s_at	6.0	.016256
Lipocalin 2, <i>Lcn2</i>	1427747_a_at	5.9	.01288
Cell division cycle 2 homolog A (<i>S. pombe</i>), <i>Cdc2a</i>	1448314_at	5.9	.000422
Cholesterol 25-hydroxylase, <i>Ch25h</i>	1449227_at	5.7	.034847
Leucine-rich alpha-2-glycoprotein 1, <i>Lrg1</i>	1417290_at	5.6	.016171
Solute carrier family 26, member 4, <i>Slc26a4</i>	1419725_at	5.5	.005928
Shc SH2-domain binding protein 1, <i>Shcbl1</i>	1416299_at	5.3	.014011
Cell division cycle associated 3, <i>Cdca3</i>	1452040_a_at	5.3	.002641
Cyclin B1, <i>Ccnb1</i>	1419943_s_at	5.2	.00567
Ribonucleotide reductase M2, <i>Rrm2</i>	1416120_at	5.1	.019074
Antigen identified by monoclonal antibody Ki 67, <i>Mki67</i>	1426817_at	5.1	.000155
Centrosomal protein 55, <i>Cep55</i>	1452242_at	5.0	.000317
Cyclin B2, <i>Ccnb2</i>	1450920_at	4.8	.000411
Ubiquitin-conjugating enzyme E2C, <i>Ube2c</i>	1452954_at	4.8	.025162
A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4, <i>Adams4</i>	1452595_at	4.8	.008109
Thymidine kinase 1, <i>Tk1</i>	1416258_at	4.6	.00906
RAD51 homolog (<i>S. cerevisiae</i>), <i>Rad51</i>	1418281_at	4.6	.000758
Spindle pole body component 25 homolog (<i>S. cerevisiae</i>), <i>Spbc25</i>	1424118_a_at	4.6	.000531
Helicase, lymphoid specific, <i>Hells</i>	1417541_at	4.5	.010822
Topoisomerase (DNA) II alpha, <i>Top2a</i>	1454694_a_at	4.4	.000008
C-type lectin domain family 4, member n, <i>Clec4n</i>	1425951_a_at	4.0	.03387

Description	Affymetrix Probe set ID	Fold change	p Value
Cell division cycle associated 1, <i>Cdca1</i>	1430811_a_at	4.0	.000807
Inter alpha-trypsin inhibitor, heavy chain 4, <i>Itih4</i>	1431808_a_at	3.9	.018824
Prostaglandin-endoperoxide synthase 2, <i>Ptgs2</i>	1417262_at	3.8	.004319
Suppressor of cytokine signaling 3, <i>Socs3</i>	1455899_x_at	3.8	.030528
Cyclin B1, related sequence 1, <i>Ccnb1-rs1</i>	1448205_at	3.7	.006735
Cell division cycle 20 homolog (S. cerevisiae), <i>Cdc20</i>	1416664_at	3.7	.007289
Ect2 oncogene, <i>Ect2</i>	1419513_a_at	3.7	.001832
Membrane targeting (tandem) C2 domain containing 1, <i>Mtac2d1</i>	1439045_x_at	3.7	.001114
Leucine zipper protein 5, <i>Luzp5</i>	1417926_at	3.6	.000143
Transforming, acidic coiled-coil containing protein 3, <i>Tacc3</i>	1455834_x_at	3.6	.00117
ASF1 anti-silencing function 1 homolog B (S. cerevisiae), <i>Asf1b</i>	1423714_at	3.6	.003282
CDC28 protein kinase 1b, <i>Cks1b</i>	1416698_a_at	3.6	.00004
Tubulin, alpha 6, <i>Tuba6</i>	1416128_at	3.6	.006892
Tumor necrosis factor (ligand) superfamily, member 9, <i>Tnfrsf9</i>	1422924_at	3.5	.003302
Fidgetin-like 1, <i>Fignl1</i>	1422430_at	3.4	.000638
Polymerase (DNA directed), epsilon, <i>Pole</i>	1448650_a_at	3.4	.005435
Methyl-CpG binding domain protein 1, <i>Mbd1</i>	1430838_x_at	3.4	.009822
Rac GTPase-activating protein 1, <i>Racgap1</i>	1451358_a_at	3.4	.002712
Ubiquitin-like, containing PHD and RING finger domains, 1, <i>Uhrf1</i>	1415810_at	3.4	.006189
Tumor necrosis factor receptor superfamily, member 12a, <i>Tnfrsf12a</i>	1418571_at	3.2	.007977
Arginase type II, <i>Arg2</i>	1418847_at	3.4	.021006
Secreted phosphoprotein 1, <i>Spp1</i>	1449254_at	3.3	.002481
Checkpoint kinase 1 homolog (S. pombe), <i>Chek1</i>	1449708_s_at	3.3	.003636
PDZ binding kinase, <i>Pbk</i>	1448627_s_at	3.3	.000095
Calcitonin/calcitonin-related polypeptide, alpha, <i>Calca</i>	1452004_at	3.3	.009521
Aldo-keto reductase family 1, member B8, <i>Akr1b8</i>	1448894_at	3.3	.000588
Serine (or cysteine) peptidase inhibitor, clade A, member 3N, <i>Serpina3n</i>	1419100_at	3.2	.000509
Kinesin family member 23, <i>Kif23</i>	1455990_at	3.2	.000845
Protein regulator of cytokinesis 1, <i>Prc1</i>	1423775_s_at	3.1	.001146
Anillin, actin binding protein (scraps homolog, <i>Drosophila</i>), <i>Anln</i>	1433543_at	3.1	.000536
Serine (or cysteine) peptidase inhibitor, clade E, member 1, <i>Serpine1</i>	1419149_at	3.0	.003037
Coagulation factor VII, <i>F7</i>	1419321 at	3.0	.000498

Description	Affymetrix Probe set ID	Fold change	p Value
Myelin and lymphocyte protein, T-cell differentiation protein, <i>Mal</i>	1417275_at	3.0	.000093
Thrombospondin 1, <i>Thbs1</i>	1460302_at	3.0	.01315
Alpha-fetoprotein, <i>Afp</i>	1416645_a_at	3.0	.001902
Cytidine 5'-triphosphate synthase, <i>Ctps</i>	1416563_at	3.0	.006285
Restin-like 2, <i>Rsl2</i>	1427278_at	3.0	.00517
ERBB receptor feedback inhibitor 1, <i>Errfi1</i>	1416129_at	3.0	.01848
Glutamate-cysteine ligase, catalytic subunit, <i>Gclc</i>	1424296_at	2.9	.001408
Budding uninhibited by benzimidazoles 1 homolog (<i>S. cerevisiae</i>), <i>Bub1</i>	1424046_at	2.9	.00066
GIN5 complex subunit 4 (Slid5 homolog), <i>Gins4</i>	1452598_at	2.9	.001311
Cathepsin K, <i>Ctsk</i>	1450652_at	2.8	.002077
Nucleolar and spindle associated protein 1, <i>Nusap1</i>	1416309_at	2.8	.00123
Early growth response 2, <i>Egr2</i>	1427683_at	2.8	.000942
Transcription factor 19, <i>Tcf19</i>	1423809_at	2.6	.001691
Growth arrest and DNA-damage-inducible 45 gamma, <i>Gadd45g</i>	1453851_a_at	2.7	.010569
Prostaglandin-endoperoxide synthase 1, <i>Ptgs1</i>	1436448_a_at	2.5	.000417
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast), <i>Mad2l1</i>	1422460_at	2.5	.002757
Karyopherin (importin) alpha 2, <i>Kpna2</i>	1415860_at	2.4	.000024
Membrane-spanning 4-domains, subfamily A, member 4C, <i>Ms4a4c</i>	1420671_x_at	-3.5	.034934
Complement receptor 2, <i>Cr2</i>	1425289_a_at	-3.4	.003537
Interferon induced transmembrane protein 6, <i>Ifitm6</i>	1440865_at	-3.3	.034152
CD19 antigen, <i>Cd19</i>	1450570_a_at	-3.1	.000444
ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide, <i>Atp1a3</i>	1427481_a_at	-3.0	.005875
Nuclear receptor subfamily 1, group D, member 1, <i>Nr1d1</i>	1426464_at	-3.0	.005349
Insulin-like growth factor binding protein 3, <i>Igfbp3</i>	1423062_at	-2.9	.001285

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Figure 1.

Plasma AT concentrations in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). ATTP deletion results in extremely low plasma AT levels ($p < .001$). There was no significant change in AT levels in O₃-exposed group as compared to their respective air-breathing littermate controls. Data are presented as mean $\pm$ SEM, $n = 5-6$.

Figure 2.

Liver AT concentrations in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). ATTP deletion results in ~35% drop of ATTP^{+/+} liver AT levels. There was no significant change in AT levels in O₃-exposed ATTP^{+/+} mice as compared to their respective air-breathing littermate controls. But a significant drop ($p < .01$) in AT levels was seen in O₃-exposed ATTP^{-/-} mice as compared to their respective air-breathing littermate controls. Data are presented as mean $\pm$ SEM, $n = 5-6$.

Figure 3. Lung AT concentrations in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). ATTP deletion results in extremely low lung AT levels ($p < .001$). There was no significant change in AT levels in O₃-exposed group as compared to their respective air-breathing littermate controls. Data are presented as mean $\pm$ SEM, $n = 5-6$.

Figure 4.

Total BAL cell count in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). A slight increase was seen in ATTP^{-/-} mice as compared to its respective air-breathing ATTP^{+/+} controls but was not statistically significant. ATTP^{-/-}/ATTP^{+/+} mice exposed to O₃ showed significant increase ($p < .001$) in BAL cell count as compared to their respective air-breathing controls. As compared to O₃-exposed ATTP^{+/+} mice, the increase in BAL cell count was statistically significant in O₃-exposed ATTP^{-/-} mice ($P < .001$) suggesting increased lung injury. Data are presented as mean $\pm$ SEM, $n = 5-6$.

Figure 5.

Total BAL protein levels in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). ATTP^{-/-}/ATTP^{+/+} mice exposed to O₃ showed significant increase ($p < .001$) in BAL protein levels as compared to its respective air-breathing controls. But no significant difference was observed in O₃-exposed ATTP^{-/-} mice as compared to O₃-exposed ATTP^{+/+} mice. Data are presented as mean $\pm$ SEM, $n = 5-6$.

Figure 6.

Venn diagram presentation of the differentially expressed genes in ATTP^{+/+} and ATTP^{-/-} mice exposed to O₃ (0.5 ppm, 6 h/day for 3 days). Fifty-two genes were differentially expressed in lungs of ATTP^{+/+} mice and 99 genes in ATTP^{-/-} mice exposed to O₃ as compared to its respective air-breathing controls. Out of these, the expression of 22 genes was common to both O₃-exposed ATTP^{+/+} and ATTP^{-/-} mice ($n = 4/\text{group}$).

Figure 7. Hierarchical clustering of genes. Each column represents an individual mouse. Each row represents a gene. Deep-red color shows high expression, green color shows low expression, and black color indicates intermediate expression of the gene.

Figure 8.

Confirmation of selected genes by qRT-PCR. The selected genes ($n = 14$) included upregulated (*Gclc*, *Saa3*, *Areg*, *Marco*, *Timp1*, *Check1*, *Cxcl2*, *Fkbp5*, *Ccna2*, *Mbd1*, *Tnc*) and downregulated genes (*Nr1d1*, *Hspb1*, *Igfbp3*). The fold-change data analyzed by qRT-PCR was similar to or lower than the microarray data and was highly correlated ($R^2 = .8076$).

Table 1

Primer sequences for real-time PCR (qRT-PCR).

Gene	Primer sequence (5'-3')	
	Sense	Anti-sense
GAPDH	TTGTGGAAGGGCTCATGACC	TCTTCTGGGTGGCAGTGATG
GCLC	GGACGCTGGAAGACCAAG	CAGGCAACTGGCGCAGA
NR1D1	CAGCGAGAAGCTCAACTCCC	AATAGGCCAGCTCCTCCTC
SAA3	AGTCATCAGCGATGCCAGAGA	GCTCCATGTCCCGTGAACCT
MARCO	CTGGGAACATCTGGCTGGAC	AACTGTTCTCTGTGCCCCGA
TIMP1	TATCCGGTACGCCTACACCC	TGGGCATATCCACAGAGGCT
CHEK1	TGGATTGAAACCAAGTTATGGG	GGGTATAAAAGCTGGCAAACCTCA
CXCL2	AACATTTTGTATGCTGGATTTCAA	TGTCCAAGGGTTACTCACAACATT
HSPB1	AAGGAAGGCGTGGTGGAGAT	TTCGTCCTGCCTTTCTTCGT
FKBP5	CTGGTCCACAGCTGGGTCTC	CGAAAGGACTTGGGAAGGCAG
IGFBP3	GCCTGGTAAGAGCATGGAGAA	AAGGACCCCAAGATCAACATACA
CCNA2	CCCAGAGGACATGTAGGCCA	GGCAGTCCTGGTAGCAAGAATT
MBD1	GTAAGCAACCGCCGCAGA	TCCTCCACCAGTGCCTCTTC

Table 2

Differentially expressed genes in lungs common to both O₃-exposed ATTP^{+/+} and ATTP^{-/-} mice as compared to their respective air-breathing controls.

Description	Affymetrix probe set ID	Fold change			p Value
		ATTP ^{+/+}	P-value	ATTP ^{-/-}	
Inflammatory-immune response					
Tissue inhibitor of metalloproteinase 1, <i>Timp1</i>	1460227_at	4.1	0.003086	13.8	.001442
Metallothionein 2, <i>Mt2</i>	1428942_at	5.7	0.039341	6.2	.005926
A disintegrin-like and metalloproteinase with thrombospondin type 1 motif 4, <i>Adamts4</i>	1452595_at	5.0	0.010648	4.8	.008109
Tumor necrosis factor receptor superfamily member 12a, <i>Tnfrsf12a</i>	1418571_at	2.8	0.006708	3.2	.007977
Serine (or cysteine) peptidase inhibitor clade E member 1, <i>Serpine1</i>	1419149_at	3.5	0.009815	3.0	.003037
Thrombospondin 1, <i>Thbs1</i>	1460302_at	3.3	0.013611	3.0	.01315
C-type lectin domain family 4 member n, <i>Clec4n</i>	1425951_a_at	5.0	0.003182	4.0	.03387
Prostaglandin-endoperoxide synthase 1, <i>Ptgs1</i>	1436448_at	2.4	0.002984	2.5	.000417
Cell proliferation/DNA repair/DNA synthesis					
Amphiregulin, <i>Areg</i>	1421134_at	5.9	0.001613	8.2	.000441
Baculoviral IAP repeat-containing 5, <i>Birc5</i>	1424278_a_at	4.7	0.011477	8.0	.001819
Cell division cycle 2 homolog A (<i>S. cerevisiae</i>), <i>Cdc2a</i>	1448314_at	5.9	0.000422	4.4	.001511
Ribonucleotide reductase M2, <i>Rrm2</i>	1416120_at	4.8	0.010085	5.1	.019074
Ubiquitin-conjugating enzyme E2C, <i>Ube2c</i>	1452954_at	3.5	0.003733	4.8	.025162
Cell division cycle associated 1, <i>Cdca1</i>	1430811_a at	5.1	0.016262	4.0	.000807
Cell division cycle 20 homolog (<i>S. cerevisiae</i>), <i>Cdc20</i>	1416664_at	5.7	0.01147	3.7	.007289
Rac-GTPase activating protein 1, <i>Racgap1</i>	1451358_a_at	3.2	0.010008	3.4	.002712
Checkpoint kinase 1 homolog (<i>S. pombe</i>), <i>Chek1</i>	1449708_s_at	4.3	0.028655	3.3	.003636
Growth arrest and DNA-damage-inducible 45 gamma, <i>Gadd45g</i>	1453851_at	3.0	0.022487	2.7	.010569
Extracellular matrix					
Tenascin C, <i>Tnc</i>	1416342_at	4.4	0.005287	8.0	.000555
Signal transduction					
Membrane-spanning 4 domains subfamily A member 4C, <i>Ms4a4c</i>	1420671_x_at	-3.8	0.007388	-3.5	.034934
Unknown function					
Similar to histone 2a, <i>MGC73635</i>	1438009_at	4.9	0.002595	6.1	.02903

Fold change					
Description	Affymetrix probe set ID	ATTTP ^{+/+}			
		ATTTP ^{+/+}	P-value	ATTTP ^{-/-}	p Value
DNA segment chr 17 human D6S56E 5, D17H6S56-E5	1417822_at	3.8	0.003788	3.5	.006364

Table 3

Differentially expressed genes in lungs of air-exposed ATTP^{-/-} compared with those in ATTP^{+/+} mice.

Description	Affymetrix probe set ID	Fold change	<i>p</i> Value
Paired related homeobox 1, <i>Prrxl</i>	1432129_a_at	-4.2	.030167
Heat-shock protein 1, <i>Hspbl</i>	1425964_x_at	-3.2	.04199

Table 4A

Selected list of upregulated genes in lungs of O₃-exposed ATTP^{+/+} mice as compared to respective air-breathing controls.

Description	Affymetrix Probe set ID	Fold change	<i>p</i> Value
Inflammatory-immune response-related genes			
FK506 binding protein 5, <i>Fkbp5</i>	1448231_at	10.9	.046047
Metallothionein 2, <i>Mt2</i>	1428942_at	5.7	.039341
Chemokine (C-X-C motif) ligand 2, <i>Cxcl2</i>	1449984_at	5.3	.018505
Tumor necrosis factor receptor superfamily, member 12a, <i>Tnfrsf12a</i>	1418571_at	2.8	.006708
Prostaglandin-endoperoxide synthase 1, <i>Ptgs1</i>	1423414_at	2.4	.002984
Cell proliferation/DNA repair/remodeling			
Cell division cycle 2 homolog A (<i>S. pombe</i>), <i>Cdc2a</i>	1448314_at	4.4	.001511
Checkpoint kinase 1 homolog (<i>S. pombe</i>), <i>Chek1</i>	1450677_at	4.3	.028655
Cell division cycle 6 homolog (<i>S. cerevisiae</i>), <i>Cdc6</i>	1417019_a_at	3.8	.019495
Transcription factors			
Period homolog 1 (<i>Drosophila</i>), <i>Per1</i>	1449851_at	3.2	.002077
Nuclear factor, interleukin 3, regulated, <i>Nfil3</i>	1418932_at	3.2	.002181
Activating transcription factor 3, <i>Atf3</i>	1449363_at	2.9	.009167
Cell-adhesion-related genes			
CEA-related cell adhesion molecule 1, <i>Ceacam1</i>	1452532_x_at	4.0	.026812
Other functions			
Iduronate 2-sulfatase, <i>Ids</i>	1450166_at	3.4	.008805
Transmembrane protein 37, <i>Tmem37</i>	1417611_at	2.9	.003164

Table 4B. Downregulated genes in lungs of O₃-exposed ATTP^{+/+} mice as compared to respective air-breathing controls.

Description	Affymetrix Probe set ID	Fold change	<i>p</i> Value
Immunoglobulin heavy chain (J558 family), <i>Igh-VJ558</i>	1427839_at	-36.2	.030697
<i>N</i> -Acylsphingosine amidohydrolase 3-like, <i>Asah3l</i>	1421496_at	-6.2	.001439
Hepatitis A virus cellular receptor 1, <i>Havcr1</i>	1450364_a_at	-5.8	.022315
Casein kappa, <i>Csnk</i>	1419735_at	-4.7	.015088
Membrane-spanning 4-domains, subfamily A, member 4C, <i>Ms4a4c</i>	1450291_s_at	-4.4	.017093
WNT1 inducible signaling pathway protein 2, <i>Wisp2</i>	1419015_at	-3.3	.013676
Protocadherin 18, <i>Pcdh18</i>	1422889_at	-2.9	.00596

Table 5A

Selected list of upregulated genes in lungs of O₃ exposed ATTP^{-/-} mice as compared to respective air-breathing controls.

Description	Affymetrix Probe set ID	Fold change	p Value
Inflammatory-immune response-related genes			
Serum amyloid A 3, <i>Saa3</i>	1450826_a_at	18.7	.008959
Metallothionein 2, <i>Mt2</i>	1428942_at	6.2	.005926
Macrophage receptor with collagenous structure, <i>Marco</i>	1449498_at	6.1	.006801
Lipocalin 2, <i>Lcn2</i>	1427747_a_at	5.9	.01288
C-type lectin domain family 4, member n, <i>Clec4n</i>	1425951_a_at	4.0	.03387
Prostaglandin-endoperoxide synthase 2, <i>Ptgs2</i>	1417262_at	3.8	.004319
Suppressor of cytokine signaling 3, <i>Socs3</i>	1455899_x_at	3.8	.030528
Tumor necrosis factor (ligand) superfamily, member 9, <i>Tnfrsf9</i>	1422924_at	3.5	.003302
Tumor necrosis factor receptor superfamily, member 12a, <i>Tnfrsf12a</i>	1418571_at	3.2	.007977
Arginase type II, <i>Arg2</i>	1418847_at	3.4	.021006
Serine (or cysteine) peptidase inhibitor, clade A, member 3N, <i>Serpina3n</i>	1419100_at	3.2	.000509
Serine (or cysteine) peptidase inhibitor, clade E, member 1, <i>Serpine1</i>	1419149_at	3.0	.003037
Coagulation factor VII, <i>F7</i>	1419321_at	3.0	.000498
Thrombospondin 1, <i>Thbs1</i>	1460302_at	3.0	.01315
Glutamate-cysteine ligase, catalytic subunit, <i>Gclc</i>	1424296_at	2.9	.001408
Prostaglandin-endoperoxide synthase 1, <i>Ptgs1</i>	1436448_a_at	2.5	.000417
Cell proliferation/DNA repair/remodeling			
Cyclin A2, <i>Ccna2</i>	1417910_at	7.2	.000661
Cell division cycle 2 homolog A (<i>S. pombe</i>), <i>Cdc2a</i>	1448314_at	5.9	.000422
Cell division cycle associated 3, <i>Cdca3</i>	1452040_a_at	5.3	.002641
Cyclin B1, <i>Ccnb1</i>	1419943_s_at	5.2	.00567
Ribonucleotide reductase M2, <i>Rrm2</i>	1416120_at	5.1	.019074
Cyclin B2, <i>Ccnb2</i>	1450920_at	4.8	.000411
RAD51 homolog (<i>S. cerevisiae</i>), <i>Rad51</i>	1418281_at	4.6	.000758
Spindle pole body component 25 homolog (<i>S. cerevisiae</i>), <i>Spbc25</i>	1424118_a_at	4.6	.000531
Cyclin B1, related sequence 1, <i>Ccnb1-rs1</i>	1448205_at	3.7	.006735
Rac GTPase-activating protein 1, <i>Racgap1</i>	1451358_a_at	3.4	.002712
Checkpoint kinase 1 homolog (<i>S. pombe</i>), <i>Chk1</i>	1449708_s_at	3.3	.003636
PDZ binding kinase, <i>Pbk</i>	1448627_s_at	3.3	.000095
Alpha-fetoprotein, <i>Afp</i>	1416645_a_at	3.0	.001902
Growth arrest and DNA-damage-inducible 45 gamma, <i>Gadd45g</i>	1453851_a_at	2.7	.010569
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast), <i>Mad2l1</i>	1422460_at	2.5	.002757
Transcription factors			

Description	Affymetrix Probe set ID	Fold change	p Value
Topoisomerase (DNA) II alpha, <i>Top2a</i>	1454694_at	4.4	.000008
Polymerase (DNA directed), epsilon, <i>Pole</i>	1448650_at	3.4	.005435
Methyl-CpG binding domain protein 1, <i>Mbd1</i>	1430838_x_at	3.4	.009822
Cytoskeleton-related genes			
Kinesin family member 22, <i>Kif22</i>	1437716_x_at	6.3	.005116
Tubulin, alpha 6, <i>Tuba6</i>	1416128_at	3.6	.006892
Kinesin family member 23, <i>Kif23</i>	1455990_at	3.2	.000845
Lipid metabolism-related genes			
Cholesterol 25-hydroxylase, <i>Ch25h</i>	1449227_at	5.7	.034847
Aldo-keto reductase family 1, member B8, <i>Akr1b8</i>	1448894_at	3.3	.000588
Ion transport-related genes			
Solute carrier family 26, member 4, <i>Slc26a4</i>	1419725_at	5.5	.005928
Unknown functions			
Resistin-like alpha, <i>Retnla</i>	1449015_at	8.4	.000305
Resistin-like 2, <i>Rsl2</i>	1427278_at	3.0	.00517

Table 5B. Downregulated genes in lungs of O₃-exposed ATP^{-/-} mice as compared to respective air-breathing controls.

Description	Affymetrix Probe set ID	Fold change	p Value
Membrane-spanning 4-domains, subfamily A, member 4C, <i>Ms4a4c</i>	1420671_x_at	-3.5	.034934
Complement receptor 2, <i>Cr2</i>	1425289_a_at	-3.4	.003537
Interferon induced transmembrane protein 6, <i>Ifitm6</i>	1440865_at	-3.3	.034152
CD19 antigen, <i>Cd19</i>	1450570_a_at	-3.1	.000444
ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide, <i>Atp1a3</i>	1427481_a_at	-3.0	.005875
Nuclear receptor subfamily 1, group D, member 1, <i>Nr1d1</i>	1426464_at	-3.0	.005349
Insulin-like growth factor binding protein 3, <i>Igfbp3</i>	1423062_at	-2.9	.001285