Published in final edited form as:

Toxicol Appl Pharmacol. 2007 July 15; 222(2): 227–234. doi:10.1016/j.taap.2007.04.010.

Lung vitamin E transport processes are affected by both age and environmental oxidants in mice[☆]

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Abstract

Despite the physiological importance of alpha-tocopherol (AT), the molecular mechanisms involved in maintaining cellular and tissue tocopherol levels remain to be fully characterized. Scavenger receptor B1 (SRB1), one of a large family of scavenger receptors, has been shown to facilitate AT transfer from HDL to peripheral tissues via apo A-1-mediated processes and to be important in the delivery of AT to the lung cells. In the present studies the effects of age and two environmental oxidants ozone (O₃) (0.25 ppm 6 h/day) and cigarette smoke (CS) (60 mg/m³ 6 h/day) for 4 days on selected aspects of AT transport in murine lung tissues were assessed. While AT levels were 25% higher ($p < 0.05$) and 15% lower ($p < 0.05$) in plasma and lung tissue, respectively, in aged versus young mice, acute environmental exposure to O₃ or CS at the doses used had no effect. Gene expression levels, determined by RT-PCR of AT transport protein (ATTP), SRB1, CD36, ATP binding cassette 3 (ABCA3) and ABCA1 and protein levels, determined by Western blots for SRB1, ATTP and ABCA1 were assessed. Aged mouse lung showed a lower levels of ATTP, ABCA3 and SRB1 and a higher level CD36 and ABCA1. Acute exposure to either O₃ or CS induced declines in ATTP and SRB1 in both aged and young mice lung. CD36 increased in both young and aged mice lung upon exposure to O₃ and CS. These findings suggest that both age and environmental oxidant exposure affect pathways related to lung AT homeostasis and do so in a way that favors declines in lung AT. However, given the approach taken, the effects cannot be traced to changes in these pathways or AT content in any specific lung associated cell type and thus highlight the need for further follow-up studies looking at specific lung associated cell types.

Keywords

Vitamin E; Lung; Ozone; Cigarette smoke; Aging

[☆]Grant support from the NIH (ESO11985), USDA (2003-00915), Actelion (VG050), MIUR (VG Brain Drain).

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Introduction

Lung tissue, exposed to near-atmospheric oxygen tension and inhaled, endogenous and circulating oxidants, is an organ routinely under oxidative stress and therefore at high risk for oxidative damage. Oxidative stress has been well documented for almost all environmental and inflammatory lung diseases (Cross et al., 1994; van der Vliet and Cross, 2000) raising the potential for antioxidant-based therapeutic strategies in some of these conditions (Rahman and MacNee, 2000; Vallyathan and Repine, 2004; Asikainen and White, 2005). Alpha tocopherol (AT) is well recognized to represent an important tissue lipophilic antioxidant micronutrient (Singh et al., 2005; Traber, 2005) although increasing emphasis is being given to its non-antioxidant functions including those related to cell signaling via PKC (Tasinato et al., 1995) and gene expression (Azzi et al., 2004; Barella et al., 2004; Gohil et al., 2004). In several studies, deficiencies of AT are recognized to increase lung tissue susceptibility to oxidative stress (Bertrand et al., 1989; Richard et al., 1990; Sinha et al., 2002). Although epidemiological studies have shown intriguing associations between micronutrient antioxidant ingestion and lung health (Kelly, 2004) including dietary intake of AT (Romieu et al., 1998; Greene, 1999; Grievink et al., 1999; Romieu and Trenga, 2001; Samet et al., 2001; Shaheen et al., 2001; Smit, 2001), to date little evidence exists showing that augmentations of AT in nutritionally replete individuals protect lung tissues from oxidative stress or ameliorate any of the lung inflammatory diseases (Cross et al., 2006).

Although, uptake liver processing and plasma distributions and whole body kinetics of AT have been explored (Traber, 2005), limited information exists concerning the uptake processes of circulating AT-specific tissues (Ricciarelli et al., 2001). The data that exist point to several non-selective and selective lipid transport processes which likely vary in their importance to AT metabolism depending on cell type (Ricciarelli et al., 2001). For example, class A scavenger receptors (SRA) may be important in macrophages (Teupser et al., 1999), CD36 in vascular smooth muscle, and SRB1 in alveolar type II cells (Kolleck et al., 2002). As HDLs are the predominant lipoproteins in most interstitial extravascular fluids (Nanjee et al., 2001), it is not surprising that HDL (apolipoprotein A1) metabolic pathways have been related to AT transport to peripheral tissue cells.

As the lung contains an abundance of different cell types, it is likely that a variety of mechanisms exist for AT transport and metabolism in this tissue. For example, it has been shown that SRB1 facilitates HDL AT uptake in lung type II cells (Kolleck et al., 2002) and that ABCA1, important in surfactant secretion, may be important in AT secretion to alveolar surfaces. Of note, it has been recently shown that lung AT levels were unaffected by the microsomal triglyceride transfer protein (Mttp) KO induced absence of apolipoprotein B containing lipoproteins, suggesting that they and their related receptors do not play significant roles in lung tocopherol delivery and homeostasis (Minehira-Castelli et al., 2006). Alveolar macrophages, an important lung cell type, possess both SRB1 and ABCA1 and thus may be active in regulating AT levels in alveolar fluids.

In the current studies, as a first step in the further characterization of lung AT transport, we have quantitated some of the AT-related transport proteins likely to be important in lung tissue AT metabolism in SKH1 mice. Interestingly, the AT transport protein (ATTP) has not yet been related to lung AT metabolism, although in the liver it is important in intracellular AT processing and insertion into secreted lipoproteins (Traber, 2005). We also explored the effects of age and two common inhaled oxidants, ozone (O₃) and cigarette smoke (CS) on the levels of these AT-related proteins using both Western blots and real-time PCR.

Methods

Animals

SKH-1 female mice (Charles River Laboratories, Wilmington, MA, USA), 8 weeks and 18 months old, were housed in individual cages in temperature- and humidity-controlled chambers (12-h light/dark cycle) with free access to tap water and standard chow diet (Harlan Tekland Rodent Diet 1846, Madison, WI, USA). Young and aged mice were allowed to acclimate for several days, randomized into groups ($N=6$) and exposed to CS or O₃ or ambient air. The animal protocol was approved by the Institutional Laboratory Animal Care and Use Committee of the University of California, Davis.

AT analysis

AT concentrations were determined by HPLC using a Waters Spherisorb ODS2 C-18 (4.6×100 mm, 3- μ m particle size) column with electrochemical detection as described by Podda et al. (1999). Tissue AT was extracted following saponification with alcoholic potassium hydroxide in the presence of 1% ascorbic acid. AT was detected electrochemically using an oxidizing potential of 500 mV and quantitated by calculation from a standard curve of authentic AT standards.

Lipoprotein profile

Lipoprotein distribution was determined by size exclusion HPLC (German et al., 1996) using a 20- μ l plasma sample on a Superose 6HR HPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). Fractions were eluted with a buffer (0.15 M NaCl, pH 7.0, and 0.02% sodium azide at a flow rate of 1.0 ml/min) and fraction total cholesterol content was determined by absorbance at 505 nm using a post-column reactor with cholesterol reagent (Boehringer-Mannheim Diagnostics, Indianapolis, IN). Cholesterol levels were calculated using a standard curve of bovine cholesterol standards (Sigma, St. Louis, MO).

CS exposure

Mice were exposed to mixture of aged and diluted sidestream and mainstream CS at a particulate concentration of 60 mg/m³ for 6 h/day for 4 days performed at the UC Davis CS facility (Pryor, 1994). Experiments were done at carbon monoxide and nicotine (mean±SD) concentrations of 198.9±5.4 ppm and 10.6±1.1 mg/m³, respectively. Food was not exposed to the CS to avoid CS induced changes in diet constituents.

O₃ exposure

Mice were exposed to O₃ in exposure chamber for 6 h/day for 4 days at 0.25 ppm continuously monitored by an O₃ detector (Dasibi model 1003-AH, Glendale, CA, USA). O₃ was produced from O₂ by electrical corona arc discharge (Sander Ozonizer model IV, Eltze, Germany) where the resulting O₂-O₃ mixture (ca. 95% O₂, 5% O₃) was mixed with ambient air and allowed to flow into a teflon-lined exposure chamber at a constant rate of 75 l/min. Exposure strategies were as previously described in detail (Valacchi et al., 2004) and food was not exposed to the O₃ to avoid O₃ induced changes in diet constituents.

Harvesting and preparation of lung samples

Tissue preparation was performed as previously detailed (Valacchi et al., 2002). Briefly, whole lungs were dissected out and then homogenized at 4 °C in RIPA buffer (150 mM NaCl₂, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1% sodium dodecyl sulfate, 5 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 10 mg/ml leupeptin, 10 μ g/ml aprotinin, 10 mg/ml iodoacetamide) (Sigma, St. Louis, MO), incubated on ice for 1

h and separated by centrifugation at 15,000 rpm for 10 min. Supernatants were stored at -80°C until further processing.

Western blot analysis

Lung homogenate samples (80 μg protein, determined using BioRad protein assay) (Bio Rad, Hercules, CA) were separated on 4–20% gradient SDS-PAGE gels and electro-transferred onto nitrocellulose membranes. After blocking in 3% non-fat milk in PBS–Tween 20, the membranes were incubated with 1 $\mu\text{g}/\text{ml}$ of specific polyclonal rabbit antibodies against either SRB1, ABCA1 (Abcam, Inc., Cambridge, MA) or ATTP (gift from Dr. Traber, LPI/OSU). Goat–anti-rabbit horseradish peroxidase-conjugated antibody (Sigma) was used as secondary antibody and after washing, bound antibodies were visualized by enhanced chemiluminescence (ECL Detection Kit, Amersham). Chemiluminescence was captured on X-ray film (Kodak) and resulting band densities were scanned and quantified using NIH image software.

Real-time quantitative PCR

Lung levels of SRB1, ABCA1, ATTP, ABCA3, CD36 and GAPDH mRNA were measured by real-time quantitative PCR using SYBER Green quantitative PCR (Perkin Elmer Applied Biosystems; Foster City, CA) and ABI Prism 7700 Sequence Detector (Perkin Elmer Applied Biosystems). Primers for SRB1, ABCA1, ATTP, ABCA3, CD36 and GAPDH, the internal control, were synthesized using Primer Express v. 1.0 software (Perkin Elmer Applied Biosystems) according to their published cDNA sequences. PCR products were measured via fluorescence and results analyzed by the Sequence Detector v1.6.3 program (Perkin Elmer Applied Biosystems). mRNA expression of the genes of interested was normalized using GAPDH mRNA levels. No-template (water) reaction mixture was used as a negative control and analyses were done in triplicate and repeated.

Statistical analysis

For each of the variables tested, two-way analysis of variance (ANOVA) was used to determine if the exposure to either CS or O_3 and age had significant effects. Data were expressed as mean $\pm$ SD of 6 mice in each treatment and a P value <0.05 was considered significant.

Results

Animal status

All animals (young and aged) appeared healthy throughout the course of the experiments and no significant differences in body weight between any of the young mice groups or between any of the aged mice groups were noted during the short-term exposure to O_3 or CS as compared to the air exposure (data not shown).

AT content

Plasma AT levels in the young and aged mice groups were 5.8 ± 0.2 mM and 7.3 ± 0.5 mM, respectively. Lung tissue AT levels in the young and aged mice groups were 78 ± 2 nmol/g tissue wet weight and 52 ± 4 nmol/g tissues wet weight respectively. Plasma AT in aged mice was statistically significantly higher (+25%, $p < 0.05$) compared to plasma AT levels in young animals. Interestingly, lung tissue AT levels in aged mice were statistically significantly lower (–30%, <0.05) compared to AT levels in lung tissue of young animals. In contrast to the significant effects of age, neither plasma nor lung tissues AT showed any statistically significant changes upon a 4-day exposure to either CS or O_3 at the levels used in the current experiments (Figs. 1A, B).

Lipoprotein profiles

Serum lipid values were measured as AT concentrations in plasma have been related to plasma lipid levels (Traber and Jialal, 2000). However, there were no differences in total cholesterol or VLDL ($19\text{--}29\pm 15$ mg/dl), LDL ($23\text{--}28\pm 6$ mg/dl) and HDL ($61\text{--}81\pm 12$ mg/dl) lipoprotein fractions between any of the animal groups (young versus old, CS or O₃ exposed).

Gene expression of SRB1, ABCA1, ABCA3 and CD36

A select number of genes related to AT trafficking were assessed in lung tissues by quantifying their mRNA levels in lung homogenates using real-time PCR. Lung SRB1 mRNA expression was lower in air exposed aged animals (-40%) compared to air exposed young animals and declined further in the aged animals after CS (33%) or O₃ (16%). Lung SRB1 mRNA levels in the young animal were affected only by O₃ exposure (60% decrease) but not by CS exposure (Fig. 2A).

In contrast to SRB1, CD36, another member of the scavenger receptor family, showed a significant increase ($+28\%$) in lung mRNA level in old animals compared to the lungs from young animals (Fig. 2B). Exposure to either CS or O₃ elicited a modest, but statistically significant, increase in lung CD36 mRNA levels in only the aged animals (Fig. 2B).

The lung expression of 2 genes in the ABCs family showed changes that differed by age and by CS and O₃ exposure. Lung ABCA1 mRNA levels were lower in young animals compared to the aged animals and increased further after CS exposure in the old animals (Fig. 2C). In contrast ABCA3 showed an opposite trend, with significantly higher mRNA levels in the air exposed young group ($+1.2$ -fold) compared to the air exposed old animals. Lung ABCA3 mRNA levels were unchanged in the old animals upon O₃ and CS exposure but declined significantly (-40%) upon O₃ exposure in young animals compared to air exposed young animals while CS exposure had no effect (Fig. 2D).

SRB1 and ABCA1 protein level

Protein level assessment using Western blots for SRB1 (Fig. 3A) shows that both O₃ and CS induced a lowering trend in lung SRB1 protein levels, which requires statistical confirmation. The declines reached statistical significance only in the O₃ exposed animals groups, with the aged animal O₃ exposed group declining the most. O₃ and CS effects on ABCA1 protein levels assessed by Western blots (Fig. 3B) showed a trend towards increased lung ABCA1 protein level upon either CS and O₃ exposure and the increase reached statistical significance compared to CS exposed young animals and air exposed aged animals in the CS exposed aged animals group.

ATTP mRNA and protein levels

Lung ATTP mRNA in the young was significantly higher ($+2$ -fold, $p<0.05$) than in the aged and moreover young and aged lung ATTP showed declines (-60% and -40% ; young and aged respectively) upon either O₃ or CS (Fig. 4A). Lung ATTP protein was detected by Western blot after IP. Lung ATTP protein was detectable in both young and aged animals, and the aged group had $\sim 30\%$ decrease of ATTP compared to the young mice. Exposure to CS or O₃ produced declines in ATTP protein level in both young and aged (-46% and -50% ; respectively; Fig. 4B), concordant with those shown by mRNA assessment.

Discussion

This study was designed to better understand AT-transport-related processes as Freed et al. (1999) had reported that anti-oxidant transport modulates airway inflammation during O₃ exposure. Young and aged mice were exposed in separate experiments to either O₃ or CS, two

representative inhaled oxidant pollutants that have significant interactions with AT (Ricciarelli et al., 2001; Richard et al., 1990). The results obtained showed: AT levels in lung tissues declined (–15%) while AT levels in plasma increased (+18%) with age; aged mice lung tissue compared to young mice lung tissue had significantly decreased ABCA3 and SRB1; while lung tissue ABCA1 and CD36 mRNA levels had an opposite trend. With respect to O₃ or CS exposure, SRB1 and ATTP as both mRNA and protein showed declines but no effects on plasma or lung tissue AT levels were apparent at the exposure times and levels used in the present studies.

The study demonstrated the presence of *ATTP* gene expression and protein in both young and aged mouse lungs, which represents a novel finding as this is the first report of the presence of this protein in the lung although others have reported the presence of ATTP mRNA in lung tissue (Hosomi et al., 1998). Lung ATTP protein levels were low, a magnitude less than found in liver (Valacchi, unpublished observation) and were only detectable via the use of immunoprecipitation as an initial step in processing the lung tissue homogenates for Western blot analysis. Moreover, the study further demonstrated that ATTP protein and mRNA levels were lower in aged animals. In addition to the decrease with age noted, acute oxidant stress as represented by either CS or O₃ caused significant declines in lung tissue ATTP as well. This behavior differs from that we reported for liver ATTP which was unaffected by CS and only modestly affected (+20–25% changes) by diets with 100-fold differences in AT levels as well as the report that 48 h of hyperoxia decreased liver ATTP mRNA while liver ATTP protein levels were unchanged (Ban et al., 2002). However, the use of whole lung homogenate precludes the localization of any specific changes to any one particular cell type.

Despite the fact that ATTP has been shown to be present in the uterus and critical for maintaining adequate AT in the developing fetus (Kaempf-Rotzoll et al., 2003), the role of ATTP in non-liver tissues remains unclear. With respect to the lung, AT is an integral constituent of surfactant which is secreted together with the other components of lung surfactant by type II cells (Rustow et al., 1993). Thus one attractive hypothesis is that ATTP, similar to its role in loading lipoproteins with AT (Qian et al., 2005), participates in loading intracellular surfactant with AT. Consistent with this role are the reports of Jang and coworkers that O₃ exposure in Balb/C mice caused alveolar fluid gamma-tocopherol content to increase (Jang et al., 2005).

The results from mice acutely exposed to either O₃ or CS demonstrated that both oxidants induced a downregulation of SRB1 in both aged and young lung tissues. In contrast, exposure to either O₃ or CS induced upregulation of *ABCA1* and CD36 genes in the lungs of both age groups. The changes in CD36 in the aged mice lungs are noteworthy as increased CD36 may have a role in increasing oxidative stress in the cells expressing this receptor (Farhangkhoei et al., 2005). Of note, CD36 has been shown to decline upon AT supplementation and increase in AT deficiency (Barella et al., 2004; Ozer et al., 2006). In the absence of any changes in lung overall AT levels, the differences in CD36 levels between the young versus the aged and the increases upon O₃ or CS suggest that signals other than AT, such as aldehydes (Viana et al., 2005) which present in CS (Reddy et al., 2002) and which can be produced by O₃ exposure (Pryor, 1994) may be responsible.

The study results also represent the first reports that lung SRB1 expression is affected by age and oxidative stress. SRB1 KO mice have AT lung levels 64% lower than controls which highlights SRB1's significant role in AT lung delivery (Rigotti et al., 2003). In the current study SRB1 declined with age and upon environmental oxidant exposure and this decline in the aged mice likely accounts for the decreased lung AT found in the aged mice. Further strengthening the relationship between SRB1 effects and AT metabolism, the aged mice decreased lung AT noted was accompanied by increased plasma AT, replicating the increased

plasma AT noted in SRB1 KO mice. The results suggest that oxidative exposure may account for the declines seen but no studies have directly examined the linkage of SRB1 with oxidative stress. However, a linkage is plausible as SRB1 expression is downregulated by the activation of the PI3-K/Akt signal transduction pathway (Cao et al., 2004) which is activated by CS and O₃ both of which were used in this study (Laskin et al., 2002; Calfee-Mason et al., 2004; Cao et al., 2004).

The current study results showed that both age and oxidant stress increased lung ABCA1. ABC transporters are linked to cholesterol in addition to AT transport and respond in a opposite fashion to changes in SRB1 (Oram, 2002). Moreover, ABCA1 knockout in mice produces lung abnormalities involving lung cholesterol content, surfactant abnormalities and impaired lung function (Bates et al., 2005). ABCA3, another member of the ABC transporter family examined in the current study, is predominantly expressed in the lung and localized to the limiting membrane of lamellar bodies in alveolar type II cells in both humans and rats (Yamano et al., 2001; Mulugeta et al., 2002). Its function is incompletely understood (Yamano et al., 2001; Mulugeta et al., 2002; Nagata et al., 2004; Shulenin et al., 2004; Bullard et al., 2005) but it appears essential for lamellar body formation as it promotes accumulation of phosphatidylcholine and inclusion of surfactant related proteins such as SP-B which is required for active surfactant surface tension lowering functions (Cheong et al., 2006). Given this, the higher levels of ABCA3 seen in the lungs of younger mice suggest a more efficient surfactant production in the young whereas the declines in ABCA3 observed in the lungs of the older animals and upon O₃ exposure suggest that aging and O₃ both may impair lung surfactant production.

We should also note that the present study represents an initial effort to characterize the interaction between oxidative stressors and aging with respect to tocopherol metabolism. As such, questions regarding the effect of age-specific changes in alveolar–capillary surface area as well as lipid (and possibly AT) levels of type II cells remain to be fully characterized. Because this study was restricted to only a few of the genes involved in the metabolism of vitamin E, more expansive studies need to completely understand the role of lung AT transport processes in the pathophysiology of lung diseases related to both age and oxidative stress.

In summary, the present study demonstrated the novel presence of TTP protein in lung tissues and documented changes in its level upon aging. This finding, as well as changes in lung AT content and the complex pattern of changes in several other AT related transport associated proteins with age, indicates that lung tissues have the complex system related to AT transport and metabolism and that components of this multifactorial system are affected by age and exposure to environmental oxidants. Of considerable potential importance, the findings show that the AT content and thus the AT related antioxidant defenses of lungs are likely to be affected by age induced declines which can be further exacerbated upon exposure to inhaled environmental oxidants. The decrease of lung vitamin E levels together with the capacity of absorb tocopherol (SRB1 decrease) can lead to a higher lung susceptibility to oxidative stress and this can consequently exacerbate existing pathologies (such as COPD and asthma) or be the trigger to such pathologies that are well associated to oxidative stress and CS. SRB1 is involved in lipid uptake essential for surfactant formation and the decrease of SRB1 expression after O₃ and CS exposure might affect surfactant composition and consequently lung functions. In addition, the study results suggest that lung-related findings based on a presumed increase in lung tocopherol upon increased plasma tocopherol levels in response to supplementation may need to be revisited as increased plasma tocopherol may not increase lung tocopherol as a result of an altered ability of the lung to take up tocopherol due to either age or oxidant stresses such as ozone and CS.

Acknowledgments

The study was supported by NIH (ESO11985), USDA (2003-00915), Actelion (VG050), MIUR (VG Brain Drain).

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Fig. 1. AT tissue concentration of (A) plasma, (B) lung from young (open bars) and old animals (closed bars) exposed to air, cigarette smoke (CS) or 0.25 ppm O₃ for 6 h/day for 4 days. Data are expressed as averages ($\pm$ SEM) of values for six mice per group. *Indicates $p < 0.05$ between young and old groups.

Fig. 2.

Lung gene expression from young (open bars) and old animals (closed bars) exposed to air, cigarette smoke (CS) or 0.25 ppm O₃ for 6 h/day for 4 days. (A) SRB1, (B) CD36, (C) ABCA1, (D) ABCA3. Data are expressed as averages ($\pm$ SEM) of ratio of gene of interest and housekeeping gene (*GADPH*) for six mice per group. *Indicates $p < 0.05$ between young and old groups. #Indicates $p < 0.05$ between control (air) and treated animal groups (CS or O₃).

Fig. 3.

In vivo exposure to CS or ozone modulated SRB1 protein expression (panel A) and ABCA1 (panel B) in murine lung. Six mice were exposed either to air, CS or to 0.25 ppm O₃ for 6 h/day for 4 days. Quantification of the SRB1, both mature (82K) and immature (66K) forms, from young (open bars) and old animals (closed bars) is shown at the bottom of panel A. Note that in panel B it is possible to discern the 3 expected bands of ABCA1, which represent ABCA1 protein modifications such as glycosylation. Quantification of the ABCA1 from young (open bars) and old animals (closed bars) is shown at the bottom of panel B. Protein levels were determined by densitometric analysis of the scanned images. All samples were normalized to protein. Data are expressed in arbitrary units and are averages of values for the six mice group.

*Indicates $p < 0.05$ between young and old groups. #Indicates $p < 0.05$ between control (air) and treated animal groups (CS or O₃).

Fig. 4.

Lung ATTP mRNA expression from young (open bars) and old animals (closed bars) exposed to air, cigarette smoke (CS) or 0.25 ppm O₃ for 6 h/day for 4 days is depicted in panel A. Data are expressed as averages ($\pm$ SEM) of ratio of gene of interest and housekeeping gene (*GADPH*) for six mice per group. Panel B depicts the quantification of the immunoprecipitated ATTP from young (open bars) and old animals (closed bars). ATTP protein level was determined by densitometric analysis of the scanned images. All samples were normalized to protein. Data are expressed in arbitrary units and are averages of values for the six mice group. A representative IP blot for lung proteins is shown at the top. *Indicates $p < 0.05$ between young

and old groups. #Indicates $p < 0.05$ between control (air) and treated animal groups (CS or O₃).