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Age-Related Differences in Cigarette Smoke Extract-Induced H₂O₂ Production by Lung Endothelial Cells

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Abstract

Cigarette smoke causes oxidative stress in the lung resulting in injury and disease. The purpose of this study was to determine if there were age-related differences in cigarette smoke extract (CSE)-induced production of reactive species in single and co-cultures of alveolar epithelial type I (AT I) cells and microvascular endothelial cells harvested from the lungs (MVECLs) of neonatal, young and old male Fischer 344 rats. Cultures of AT I cells and MVECLs grown separately (single culture) and together (co-culture) were exposed to CSE (1, 10, 50, 100%). Cultures were assayed for the production of intracellular reactive oxygen species (ROS), hydroxyl radical (OH[•]), peroxynitrite (ONOO⁻), nitric oxide (NO) and extracellular hydrogen peroxide (H₂O₂). Single and co-cultures of AT I cells and MVECLs from all three ages produced minimal intracellular ROS in response to CSE. All ages of MVECLs produced H₂O₂ in response to CSE, but young MVECLs produced significantly less H₂O₂ compared to neonatal and old MVECLs. Interestingly, when grown as a co-culture with age-matched AT I cells, neonatal and old MVECLs demonstrated ~50% reduction in H₂O₂ production in response to CSE. However, H₂O₂ production in young MVECLs grown as a co-culture with young AT I cells did not change with CSE exposure. To begin investigating for a potential mechanism to explain the reduction in H₂O₂ production in the co-cultures, we evaluated single and co-cultures for extracellular total antioxidant capacity. We also performed gene expression profiling specific to oxidant and anti-oxidant pathways. The total antioxidant capacity of the AT I cell supernatant was ~5 times greater than that of the MVECLs, and when grown as a co-culture and exposed to CSE (≥ 10%), the total antioxidant capacity of the supernatant was reduced by ~50 %. There were no age-related differences in total antioxidant capacity of the cell supernatants. Gene expression profiling found eight genes to be significantly up-regulated or down-regulated. This is the first study to describe age-related differences in MVECLs exposed to CSE.

Keywords

endothelial cell; alveolar type I cell; hydrogen peroxide; oxidative stress; rat

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Introduction

Smoking and cigarette smoke cause disease; however, despite the well-publicized harmful effects of cigarette smoke, nearly 46 million Americans continue to smoke (CDC, 2011). In addition, studies demonstrate the harmful effects of second-hand and third-hand smoke. For example, second-hand smoke, the inhalation of smoke from tobacco products used by others, has been implicated in the development of low birth-weight infants, Sudden Infant Death Syndrome (SIDS) (Pershagen, 1986), as well as cardio-pulmonary disease and cancer (Waris & Ahsan, 2006; Precioso, Samorinha, Celheiros et al., 2010). The effects of third-hand smoke, particles deposited in the environment after a cigarette has been extinguished, have only recently been reported. Studies are linking the development of cardio-pulmonary disease, especially among infants and children, to third-hand smoke exposure (Becquemin, Bertholon, Bentayeb et al 2010; Yang, Kong, Zhao et al, 2010). Collectively these studies suggest that the effects of cigarette smoke are more far reaching, and devastating, especially in the very young, than previously thought. Consequently it is important to understand the effects of cigarette smoke and to determine what role age may play in cigarette smoke-induced injury in order to improve preventative strategies and therapeutic interventions.

It is known that cigarette smoke results in oxidative stress, an imbalance between oxidants and antioxidants in favor of oxidants, and that it activates the vascular endothelium (Halliwell & Gutteridge, 2007). The activated endothelium favors inflammation, a hallmark feature of COPD. Multiple studies describe an increase in macrophages, monocytes that have migrated from the vascular space into alveolar space, in the lungs of smokers and individuals with COPD (Willemse, Ten Hacken, Rutgers et al., 2002). Macrophages produce ROS that cause oxidative stress leading to apoptosis, senescence, and inflammation—all of which have been described in the airways of smokers with COPD (Aoshiba, Yokohori & Nagai, 2003; Tsuji, Aoshiba & Nagai, 2004, 2006). Ultimately, cigarette smoke affects the lung leading to oxidative stress, inflammation and disease.

Despite the advances made in understanding the cellular and molecular processes involved in the pathogenesis of COPD, much remains unknown about the effects of cigarette smoke on the alveolus, the gas exchanging unit of the lung. The alveolus is composed of alveolar type I (AT I) cells, alveolar type II (AT II) cells and endothelial cells. AT I cells are large, flat squamous epithelial cells that cover ~95% of the alveolar surface area and are responsible for gas, ion and water exchange (Crapo, Barry, Gehr et al., 1982; Helms, Jain, Self et al., 2008). AT II cells are small cuboidal cells that produce surfactant, a lipoprotein substance that reduces surface tension in the alveolus. Cigarette smoke is known to induce apoptosis in the cells of the alveolus leading to the development of emphysema, a common form of COPD. We have previously described the development of cell culture models using rat primary AT I cells and MVECLs that can be used to investigate the effects of cigarette smoke extract (Downs, Montgomery & Merkle, 2011).

Cigarette smoke is known to activate the endothelium in cell culture models. However, it is not known how AT I cells respond to cigarette smoke, nor is it known if there is an interaction effect between AT I cells and MVECLs when grown as a co-culture and exposed to cigarette smoke. Furthermore, it is not known if age influences or contributes to the responses of MVECLs and AT I cells. Therefore we performed this study to begin describing the responses of AT I cells and MVECLs to cigarette smoke by exposing single and co-cultures of AT I and MVECLs harvested from the lungs of neonatal, young and old male rats to cigarette smoke extract (CSE).

In the current study we exposed cultures of AT I cells and MVECLs grown separately (single) and together (co-culture) to CSE and quantified ROS and reactive nitrogen species

production, here after referred to as reactive species. We observed age-related differences in CSE-induced H₂O₂ production by MVECLs. Furthermore, we observed up to a 50% reduction in CSE-induced production of H₂O₂ in co-cultures. The cell supernatant from cultures containing AT I cells demonstrated greater total antioxidant capacity compared to MVECLs alone and this observation may partially explain the reduction in H₂O₂ production observed in the co-cultures. Gene expression profiling specific to oxidant and antioxidant pathways identified eight genes that were up-regulated or down-regulated in MVECLs grown as a co-culture with AT I cells compared to MVECLs alone. We present several novel findings. First, this is the first study to describe age-related differences in CSE-induced production of H₂O₂ in MVECLs. Second, when grown as a co-culture, the quantity of H₂O₂ produced in response to CSE decreased, suggesting an interaction effect between MVECLs and AT I cells. Third, we observed that the supernatants of AT I cells contained greater total antioxidant capacity compared to MVECLs which may explain the reduction in H₂O₂ in the co-cultures as well as the minimal production of reactive species by AT I cells when challenged with CSE.

Materials and methods

Animals

Fischer 344 rats (NIA-NIH aging colony) were purchased from Harlan Laboratories (Indianapolis, IN) and used as the source for MVECLs and AT I cells. Three age groups (neonatal, young and old) of male Fischer 344 rats were used. Fischer 344 dams were used as the source for neonatal pups (7 day old); the gender of neonatal pups was determined following euthanasia by making a small incision in the lower abdomen to look for undescended testes. Young rats were 3 months old and old rats were 24 months old. Protocols for animal use were approved by the Institutional Animal Care and Use Committee of The University of Arizona and The Southern Arizona VA HealthCare System.

Reagents, kits and antibodies

Unless stated otherwise all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). The Amplex Red Hydrogen Peroxide Detection kit and CM-H₂DCFDA kit were purchased from Invitrogen (Carlsbad, CA). The OH[•]/ONOO⁻ Detection kit was purchased from Cell Technology (Mountain View, CA). The Total Antioxidant Capacity kit and RPMI 1640 media were purchased from Sigma-Aldrich (St. Louis, MO). The CellTiter[®] AQueous One Solution Assay, an MTS assay, was purchased from Promega (Madison, WI). The RNeasy mini kit, RT² First Strand kit and the Oxidant and Antioxidant Defense gene array were purchased from Qiagen (Frederick, MD).

Cell culture models

MVECLs and AT I cells were isolated from the lungs of neonatal, young and old male Fischer 344 rats. In brief, the lung tissue was harvested and enzymatically digested, then magnetic beads coated with antibodies specific to cell surface proteins were used to positively select for either MVECLs or AT I cells. Neonatal, young and old AT I cells and MVECLs were cultured separately (single culture) and together (co-culture). Single culture systems consisted of MVECLs and AT I cells grown separately while the co-cultures were created by seeding MVECLs and AT I cells on opposite sides of fibronectin coated 24 well Transwell Fluroblok[™] filters. Cell cultures were maintained in RPMI 1640 with 10% FBS; medium was changed every other day.

Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was standardized by bubbling the smoke from one 1R5F research grade cigarette (1.67 mg tar, 0.16 mg nicotine, and 2.08 mg total particulate matter per cigarette; The University of Kentucky, Lexington, KY) into 10 mL of RPMI 1640 with 10% FBS over 3 minutes using a cigarette smoking apparatus. The CSE was pH corrected (7.4), then filter sterilized and the absorbance value read at 320nm using a Tecan GENios plate reader (Basil, Switzerland), and only CSE preparations with an absorbance value of 0.42 ± 0.03 were used. The resulting CSE was known as 100% CSE and used at lower concentrations (1, 10, and 50%) by diluting 100% CSE in RPMI 1640 with 10% FBS. Vehicle control medium was made by bubbling air through RPMI 1640 with 10% FBS for three minutes followed by filter sterilizing.

Experimental setup and design

Unless stated otherwise, all experiments adhered to the following experimental design. Three age groups of AT I cells and MVECLs (population doubling range of 4–8) harvested from neonatal (7 day), young (3 months) and old (24 months) Fischer 344 rats were used concurrently in all experiments. In the single cultures, AT I cells and MVECLs were seeded to confluence (separately) at a known density (2.0×10^5) on gelatinized 96 well microtiter plates. In the co-culture experiments, AT I cells (2.0×10^5) were seeded (approximately 80 % confluence) on the top and MVECLs (2.0×10^5) on the bottom surfaces of fibronectin coated 24 well Transwell Fluoroblok™ filters. Cells were maintained in RPMI 1640 with 10% FBS for 24 hours after seeding to allow confluency to be achieved. The next day, cells were exposed to CSE (1, 10, 50 and 100%) or vehicle control for 24 hours and assayed as described below. All fluorescence intensity and absorbance measurements were performed using a plate reader.

Intracellular reactive species production

Intracellular ROS production was measured using the CM-H₂DCFDA kit according to the manufacturer's instructions. The kit detects hydrogen peroxide, hydroxyl radical, peroxy radical and peroxynitrite. Briefly, the cells were labeled with the CM-H₂DCFDA probe and incubated at 37° C with 5% CO₂/air for 1 hour. Following incubation with the CM-H₂DCFDA probe, the cells were exposed to various concentrations of CSE (1,10,50 and 100%) for 1 hour, the culture medium removed and the cells rinsed twice with Krebs's Ringers and the fluorescence intensity measured (excitation/emission 485/535 nm).

Intracellular nitric oxide (NO), peroxynitrite (ONOO⁻) and hydroxyl radical (OH[•]) production in CSE exposed MVECLs was measured following 24 hours of CSE exposure. To detect NO production the DAF-FM diacetate kit was used according to the manufacturer's instructions and the fluorescence intensity read (excitation/emission 485/535 nm). To detect OH[•]/ONOO⁻ production, the OH[•]/ONOO⁻ detection kit was used according to the manufacturer's instructions and the fluorescence intensity measured (excitation/emission 495/515 nm).

Hydrogen peroxide production

Hydrogen peroxide (H₂O₂) production in response to CSE was measured from single and co-cultures of MVECLs and AT I cells after CSE addition. H₂O₂ production was measured using the Amplex Red hydrogen peroxide detection kit according to the manufacturer's instructions. Briefly, cells were exposed to CSE for 24 hours, then removed and Krebs's Ringers added for 4 hours, then aliquots of the cell supernatant were removed and incubated with the Amplex Red horseradish peroxidase reagent solution for 4 hours at 37° C. In the co-culture experiments the cell supernatant (Krebs's) from the upper and lower chambers were

thoroughly mixed and then transferred to a 96 well plate for incubation with the Amplex Red horseradish peroxidase reagent. Following incubation the fluorescence intensity was measured (excitation/emission 530/590 nm).

Antioxidant capacity of MVECLs and AT I cells supernatant from single and co-cultures

To determine the antioxidant capacity of the cell supernatant from single and co-cultures of MVECLs and AT I cells, the Total Antioxidant Capacity kit was used according to the manufacturer's instructions. Following 24 hours of exposure to CSE, the media were aspirated and flash frozen in liquid nitrogen and stored at -80°C until assayed. The principle underlying the antioxidant assay is the formation of a radical which oxidizes the ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to a radical cation, $\text{ABTS}^{\bullet+}$, that can be measured spectrophotometrically at 405 nm. The assay uses Trolox, a vitamin E analogue, to create a standard curve which is then used to determine total antioxidant capacity of the samples.

Relative cell number

To normalize the production of intracellular ROS and hydrogen peroxide production to cell number, the relative cell number of AT I cells and MVECLs in the single and co-culture models was determined using the Cell Titer[®] AQueous One Solution Assay, an MTS assay. Single and co-cultures of AT I cells and MVECLs were treated concurrently with samples used in the intracellular ROS and hydrogen peroxide assays described below. The cells were then labeled with the Cell Titer[®] AQueous One Solution and incubated at 37°C with 5% CO_2 /air for 1 hour and the optical density (O.D.) at 492 nm read. The O.D. values of cell cultures following 24 hours exposure to CSE did not show a change in relative cell number compared to control cultures.

PCR and gene expression array

Single and co-cultures of AT I cells and MVECLs were exposed to 10% CSE to evaluate for gene expression changes. Following 24 hours exposure to CSE, AT I and MVECLs were rinsed with ice cold PBS, scraped from the filter and cells flash frozen in liquid nitrogen. RNA was isolated using the RNeasy mini kit according to the manufacturer's instructions. RNA quality and quantity was determined by first assessing $A_{260/280}$ (only samples with values ≥ 2.0 were used), and then samples were run on a 1.2% denaturing RNA gel. cDNA synthesis was performed using the RT² First Strand Kit, and cDNA added to the rat oxidant and antioxidant defense RT²PCR gene array. The gene array contains 86 genes involved in antioxidant production, ROS metabolism and oxygen transportation. Real time quantitative PCR was performed using an ABI 7300 thermocycler (Applied Biosciences, Carlsbad, CA). Cycle to threshold (C_t) data analysis was performed using the ABI 7300 sequence detection software.

Statistical analysis

Student's t-test was performed to assess for differences in gene expression. In all other experiments multiple comparisons were performed using two way analysis of variance (ANOVA), F, followed by Dunn's method for all post hoc pairwise comparisons. Probability values of less than 0.05 were considered significant. Data presented below are expressed as means $\pm$ SEM.

Results

Age-related differences in CSE-induced H₂O₂ production by MVECLs

MVECLs exposed to a range of CSE concentrations (0, 1, 10, 50 and 100%) show a dose dependent increase in H₂O₂ ($F(2,8) = 29.55$, $p < 0.001$) with CSE-exposure increasing H₂O₂ concentrations as much as 100% ($F(2,8) = 4.17$, $p < 0.001$). There was an interaction between age and CSE concentration ($F(2,8) = 5.18$, $p < 0.001$) (Figure 1A) with young MVECLs producing less H₂O₂ compared to the other two groups. There was no change in H₂O₂ production by AT I cells in response to CSE, nor was there an effect related to age (Figure 1B). Interestingly, when grown as a co-culture (Figure 1C), the concentration of CSE-induced H₂O₂ decreased by nearly 50% compared to single cultures alone. Exposure to 100% CSE significantly increased ($p < 0.001$) H₂O₂ production in neonatal and old cells compared to young cells. Therefore demonstrating that young MVECLs and AT I cells respond differently to the addition of CSE compared to neonatal and old cells. In addition, the data suggest that when grown as a co-culture all age groups of MVECLs and AT I cells demonstrate an interaction effect. Surprisingly, we did not observe a significant increase in intracellular ROS production in either MVECLs or AT I cells exposed to CSE (Figure 2A, B). However, we did observe age-related differences in ROS production following CSE exposure. In single and co-cultures, young MVECLs and neonatal AT I cells produced less ROS following exposure to CSE. Collectively, these observations suggest that there are age-related differences in CSE-induced production of reactive species in rat primary MVECLs and AT I cells.

MVECLs exposed to a range of CSE concentrations (0, 1, 10, 50 and 100%) for 24 hours demonstrated up to 100% increase in NO production with 100% CSE exposure ($F(2,8) = 24.25$, $p < 0.001$) (Figure 3A). However, there was no effect related to age, nor was there an interaction effect between age and CSE concentration. Furthermore, there was no difference when MVECLs were grown as a co-culture with AT I cells (data not shown). Similarly, MVECLs exposed to CSE demonstrate up to 230% increase in the production of ONOO⁻ and OH[•] ($F(2,8) = 38.82$, $p < 0.001$) (Figure 3B), but failed to reveal any age related differences or an interaction effect between age and CSE concentration. Also, there was no difference in the production of ONOO⁻ or OH[•] when MVECLs were grown as a co-culture with AT I cells (data not shown) suggesting that CSE exposure increases the production of NO, ONOO⁻ and OH[•] in MVECLs independent of the presence of AT I cells and that their production is stable across age groups.

Changes in antioxidant capacity of cell supernatants occurs after exposure to CSE

To begin determining the mechanism by which the reduction in H₂O₂ occurred, MVECLs and AT I cells were grown as single and co-cultures and exposed to vehicle control or a range of CSE concentrations (0,1,10,50 100%) and then the cell supernatants assayed to determine total antioxidant capacity. The total antioxidant capacity of MVECLs supernatant (Figure 4A) was approximately 1/8 as potent as the supernatant of AT I cells (Figure 4B). The antioxidant capacity of the MVECLs supernatant increased by up to 300% with CSE exposure ($F(2,8) = 7.37 \times 10^{15}$, $p < 0.001$) while the total antioxidant capacity of the neonatal cell supernatant decreased nearly 500% with 100% CSE exposure ($F(2,8) = 3.78 \times 10^{16}$, $p < 0.001$). There was an interaction effect between cell age and CSE exposure at CSE concentrations of $\geq 10\%$ ($F(2,8) = 5.44 \times 10^{15}$, $p < 0.001$).

The total antioxidant capacity of AT I cell supernatants decreased with CSE exposure ($F(2,8) = 3.56$, $p < 0.05$). Furthermore, the cell supernatant from neonatal AT I cells demonstrated lower antioxidant capacity compared to the other two groups ($F(2,8) = 40.73$, $p < 0.001$) and there was an interaction effect between CSE and cell age ($F(2,8) = 3.20$, $p <$

0.05). Surprisingly, when grown as a co-culture (Figure 4C), there was nearly a 400% reduction in total antioxidant capacity of cells exposed to CSE compared to controls ($F(2,8) = 1.0 \times 10^{20}$, $p < 0.001$) with the supernatant from young cells differing from the other two groups ($F(2,8) = 1.0 \times 10^{20}$, $p < 0.001$). There was an interaction effect between age and CSE concentration ($F(2,8) = 1.0 \times 10^{20}$, $p < 0.001$). These findings suggest that there are differences in the total antioxidant capacity of MVECLs and AT I cells, and that CSE exposure reduces the total antioxidant capacity of co-cultures across ages.

Next we assayed single and co-cultures of AT I cells and MVECLs exposed to 10% CSE to evaluate changes in genes involved in the production of oxidants and in antioxidant defense. Gene expression profiling of AT I cells did not reveal significant differences in the response to CSE from AT I cells grown separately or as a co-culture with MVECLs. However, eight genes were found to be significantly up-regulated or down-regulated (Table 1) in MVECLs grown as a co-culture compared to MVECLs in single culture. These genes include *ApoE*, *Cygb*, *Ercc6*, *Nqo1*, *Prnp*, *Clf38a1*, *Tmod1* and *Zmynd17*. These genes may provide clues into anti-oxidant pathways that may be up-regulated or oxidant producing pathways that are down-regulated to explain the reduction in CSE-induced production of H_2O_2 by MVECLs observed when MVECLs were grown as a co-culture with AT I cells.

Discussion

Cigarette smoke is a complex mixture of over 4700 chemicals including free radicals such as quinones, organic molecules such as aldehydes, and nitrosamines (Church & Pryor, 1985). Cigarette smoke and cigarette smoking are known to result in oxidative stress and inflammation (Faux et al., 2009). Cigarette smoking is known to cause COPD, a form of chronic lung disease that is characterized by abnormal and persistent inflammation, and data strongly implicate a role of oxidative stress in the pathology of COPD (Pauwels et al., 2004). COPD typically occurs in the fourth and fifth decade of life which suggests an aging component; however, cigarette smoke and cigarette smoking, through second and third-hand smoke exposure, also cause disease (Precioso et al., 2010). In the current study we aimed to investigate age-related differences in reactive species production in cultured rat primary AT I cells and MVECLs. During our investigation we observed several important findings that warrant additional discussion.

First, we observed age-related differences in CSE-induced production of reactive species in MVECLs grown as single and co-cultures with age-matched AT I cells. In the single cultures, each age group of MVECLs increased production of H_2O_2 in response to CSE with neonatal cells producing the most and young cells the least. When grown as a co-culture with age-matched AT I cells, CSE-induced H_2O_2 by neonatal and old cells decreased while young cells did not produce H_2O_2 in response to CSE. Young MVECLs also produced less intracellular ROS although exposure to CSE failed to result in a significant increase in ROS production in all age groups. Collectively these data suggest that young cells respond differently to CSE than neonatal and old cells. To our knowledge this is the first study to describe age-related differences in CSE-induced production of reactive species which may provide insight into the role of age in human health and disease, specifically in lung diseases where age and/or aging plays a role such as asthma and COPD.

Oxidative stress plays a role in the development of many lung diseases. However, age-related differences in the oxidative state of the lung are not known. It is known that H_2O_2 serves as a signaling molecule to trigger a wide variety of cellular processes such as proliferation (Burdon, Alliangana & Gill, 1995), apoptosis (Antunes & Cadenas, 2001), and inflammation (Bokoch & Knaus, 2003). In our hands, the basal production of H_2O_2 did not significantly vary between single and co-cultures. This suggests that basal H_2O_2

concentrations are consistent in *in vitro* models composed of single and co-cultures of primary AT I cells and MVECLs and that basal H₂O₂ concentrations are not summative. This has implications for understanding the role of H₂O₂ in cell-to-cell communication in the alveolus, specifically in the communication between the pulmonary epithelium and endothelium—e.g. potentially, small changes in H₂O₂ concentrations might trigger H₂O₂ mediated events. However, more work is needed to address this issue and future studies are planned to better describe the role of H₂O₂ in cell-to-cell communication between the pulmonary epithelium and endothelium.

Second, we observed that AT I cells exposed to CSE produced minimal intracellular ROS or extracellular H₂O₂. This was unexpected because many cell types have been described to produce intracellular ROS and H₂O₂ in response to CSE. For example, mammalian AT II cells (Jorgensen et al., 2008), fibroblasts (Bagloli et al, 2006) and A549 cells (Jiao et al., 2006) are known to increase intracellular ROS production in response to CSE. In addition, endothelial cells, as shown by others, produce ROS in response to CSE (Nana-Sinkam et al., 2007). Furthermore, low concentrations of CSE have been reported to result in an increase in H₂O₂ production in A549 cells (Liu et al., 2010), but the response of primary AT I cells to CSE is unknown. The lack of intracellular ROS production by rat primary AT I cells exposed to CSE suggests that AT I cells respond differently than other cell types in the alveolus. Under hyperoxic conditions, rat AT I cells were found to be protective against oxidative injury (Chen et al., 2006). We observed a reduction in the total antioxidant capacity of the CSE-exposed supernatants from cultures containing AT I cells, which suggests utilization of extracellular antioxidants in the cell supernatant and supports a protective role of the AT I cell in protecting against oxidative injury. Also, AT I cell supernatants contained significantly greater concentrations than MVECLs. These observations may have significance for understanding the role of AT I cells in the formation of the epithelial lining fluid, a thin lining of antioxidant rich fluid that protects the alveolar epithelium from the continuous bombardment of oxygen and its radicals (Cross, van der Villiet, O'Neill et al., 1994).

Third, we tested a “stop-or-mop” hypothesis in which intracellular production of oxidants was either stopped or oxidants were mopped up by antioxidants by performing gene expression profiling arrays of MVECLs grown as single and co-cultures with age-matched AT I cells. To evaluate for a mop mechanism, we also assayed the cell supernatant of single and co-cultures of MVECLs and AT I cells for total antioxidant capacity.

AT I cells did not demonstrate a significant change in any of the 84 genes studied when grown separately or as a co-culture with MVECLs. Furthermore, there was a mixed response in the expression of genes involved in ROS metabolism, oxidative stress response, and oxygen transport. This suggests that AT I cells respond similarly to the addition of CSE when grown as a single culture as well as when grown as a co-culture with MVECLs. Moreover a one-time challenge with CSE did not significantly affect the transcription of genes surveyed in the oxidant and antioxidant defense array.

Eight genes were found to be significantly up- or down-regulated in MVECLs grown as a co-culture compared to MVECLs grown as a single culture (Table 1). Two genes, *Cygb* and *Slc38a1*, encode proteins involved in oxygen transport and both of these genes were down-regulated. *Cygb* is an oxygen transport protein and plays a role in scavenging ROS and NO. *Slc38a1* is involved in cellular metabolism of amino acids, a process that produces ROS. Down regulation of these genes may signify a compensatory response leading to a reduction in ROS, a potential stopping mechanism. *Ercc6*, *Zymnd17* and *Nqo1* were up-regulated. *Ercc6* is involved in repair of damaged DNA, *Zymnd17* plays a role in the stabilization of proteins such as DNA, and *Nqo1* plays an important role in the reduction of quinones to

hydroquinones. The Nqo1 enzyme prevents the production the one electron reduction of quinones that results in the production of radical species, such as superoxide. Several other genes, ApoE, Prnp and Tmod1, were down-regulated. ApoE is involved in the antioxidant response although its exact role is unclear. ApoE is secreted by AT I cells and has been shown to be protective against hyperoxia-induced oxidative stress in animal models (Chen et al., 2006). Prnp is involved in apoptosis as well as the antioxidant response, although the mechanism is not understood. Tmod1 plays a role in antioxidant defense; reportedly the Tmod1 enzyme possesses peroxidase activity. Many genes did not demonstrate a significant change and include catalase, glutathione peroxidases (1–6), peroxidases (1–6) and superoxide dismutases (1–3). Data from these gene expression profiling studies do not conclusively point to a stopping mechanism—e.g. down-regulation in pathways where ROS are a by-product—to explain the reduction in H₂O₂ production observed in CSE exposed co-cultures. Rather the data provide clues into potential pathways that can be explored in future studies to better understand and characterize potential signaling mechanisms between the alveolar epithelium and endothelium.

We did observe that AT I cell supernatant contained 6 to 8 times greater antioxidant capacity compared to MVECLs. Furthermore, following exposure to CSE, the total antioxidant of cell cultures containing AT I cells was significantly reduced. However, we did not observe an increase in gene expression of antioxidants in AT I cells or MVECLs. It is then plausible that the reduction in CSE-induced H₂O₂ by MVECLs could occur due to a mopping effect of antioxidants in the cell supernatant from the co-cultures.

In summary, we report several novel observations. First, there are age-related differences in the response of MVECLs and AT I cells to CSE. Second, we report that CSE-induced production of H₂O₂ is significantly reduced when MVECLs are grown as a co-culture with AT I cells compared to single cultures alone, suggesting an interaction effect. Third, the reduction in CSE-induced production of H₂O₂ may have occurred due to antioxidants in the cell supernatant ‘mopping-up’ oxidants, though ‘stopping’ mechanisms cannot be excluded. Finally, data from gene expression profiling may provide clues into signaling pathways that may exist between the alveolar epithelium and endothelium that can be studied to better understand the pathogenesis of cigarette smoke-related diseases. Collectively these observations inform our understanding of the age-related effects of CSE-induced production of reactive species in alveolar epithelial and endothelial cells that may provide insight into CSE-induced oxidative injury in the lung.

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References

- Antunes F, Cadenas E. Cellular titration of apoptosis with steady state concentrations of H₂O₂: submicromolar levels of H₂O₂ induce apoptosis through Fenton chemistry independent of the cellular thiol state. *Free Radic Biol Med.* 2001; 30:1008–1018. [PubMed: 11316581]
- Aoshiba K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol.* 2003; 25:555–562. [PubMed: 12707011]
- Bagloli CJ, Bushinsky SM, Garcia TM, et al. Differential induction of apoptosis by cigarette smoke extract in primary human lung fibroblasts strains: implications for emphysema. *Am J Physiol Lung Cell Mol Physiol.* 2006; 291:L19–L29. [PubMed: 16443644]

- Becquemin MH, Bertholon JF, Bentayeb M, et al. Third-hand smoking: indoor measurements of concentration and sizes of cigarette smoke particles after resuspension. *Tob Control*. 2010; 19:347–348. [PubMed: 20530137]
- Bokoch GM, Kanus UG. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem Sci*. 2003; 28:502–508. [PubMed: 13678962]
- Burdon RH, Alliangana D, Gill V. Hydrogen peroxide and the proliferation of BHK-21 cells. *Free Radic Res*. 1995; 23:471–486. [PubMed: 7581830]
- CDC. Adult cigarette smoking in the United States: current estimate. 2011. http://www.cdc.gov/tobacco/data_statistics/fact_sheets/adult_data/cig_smoking/index.htm
- Church DF, Pryor WA. Free radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect*. 1985; 64:111–126. [PubMed: 3007083]
- Chen J, Chen Z, Chintagari N, et al. Alveolar type I cells protect rat lung epithelium from oxidative injury. *J Physiol*. 2006; 572:625–638. [PubMed: 16497717]
- Crapo JD, Barry BE, Gehr P, et al. Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis*. 1982; 126:332–337. [PubMed: 7103258]
- Cross CE, van der Vliet A, O'Neill CA, et al. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect*. 1994; 10:185–191. [PubMed: 7705296]
- Downs CA, Montgomery DW, Merkle CJ. Cell culture models using rat primary alveolar type I cells. *Pulm Pharmacol Toxicol*. 2011; 24:577–86.
- Faux SP, Tai T, Thorne D, et al. The role of oxidative stress in the biological responses of lung epithelial cells to cigarette smoke. *Biomarkers, Suppl*. 2009; 1:90–96.
- Folch E, Salas A, Prats N, et al. H₂O₂ and Pars mediate lung p-selectin upregulation in acute pancreatitis. *Free Rad Biol Med*. 2000; 28:1286–1294. [PubMed: 10889459]
- Halliwell, B.; Gutteridge. *Free radicals in biology and medicine*. 4. Oxford University Press; New York, NY: 2007.
- Hanssen TA, Jorgensen L. Obstruction of the lung capillaries by blood platelet aggregates and leucocytes in sudden infant death syndrome. *APMIS*. 2010; 118:958–967. [PubMed: 21091777]
- Helms MN, Jain L, Self JL, et al. Redox regulation of epithelial sodium channels examined in alveolar type I and 2 cells patch clamped in lung slice tissue. *J Biol Chem*. 2008; 283:22875–22883. [PubMed: 18541535]
- Jiao ZX, Ao QL, Xiong M. Cigarette smoke extract inhibits the proliferation of alveolar epithelial cells and induces apoptosis. *Sheng Li Xue Bao*. 2006; 25:244–254. [PubMed: 16786109]
- Liu Y, Gao W, Zhang D. Effects of cigarette smoke extract on A549 cells and human lung fibroblasts treated with transforming growth factor β 1 in a coculture system. *Clin Exp Med*. 2010; 10:159–167. [PubMed: 19924381]
- Nana-Sinkam SP, Lee JD, Sotto-Santiago S, et al. Prostacyclin prevents pulmonary endothelial cell apoptosis induced by cigarette smoke. *Am J Respir Crit Care Med*. 2007; 175:676–685. [PubMed: 17255567]
- Okayama N, Coe L, Itoh M, Alexander JS. Intracellular mechanisms of nitric oxide plus hydrogen peroxide mediated neutrophil adherence to cultured human endothelial cells. *Inflamm Res*. 1998; 47:428–433. [PubMed: 9865501]
- Pauwels RA, Buist AS, Ma P, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. *Respir care*. 2004; 46(8):795–825.
- Precioso J, Samorinha C, Calheiros JM, et al. Second hand smoke (SHS) exposure in children. An evaluation of a preventative measure. *Rev Port Pneumol*. 2010; 16:57–72. [PubMed: 20054508]
- Pershagen. Review of epidemiology in relation to passive smoking. *Arc Toxic Suppl*. 1986; 9:63–73.
- Rahman I, Li XY, Donaldson K, et al. Glutathione homeostasis in alveolar epithelial cells in vitro and lung in vivo under oxidative stress. *Am J Physiol*. 1995; 269:L285–292. [PubMed: 7573460]
- Tsuji T, Aoshiba K, Nagai A. Cigarette smoke induces senescence in alveolar epithelial cells. *Am J Respir Cell Mol Biol*. 2004; 31:643–649. [PubMed: 15333326]

- Tsuji T, Aoshiba K, Nagai A. Alveolar cell senescence in patients with pulmonary emphysema. *Am J Respir Cell Mol Biol.* 2006; 174:643–649.
- Waris G, Ashan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog.* 2006; 5:14. [PubMed: 16689993]
- Watnabe N, Dickinson DA, Liu RM, et al. Quinones and glutathione metabolism. *Method Enzymo.* 2004; 378:319–340.
- Willemse BWM, Ten Hacken NH, Rutgers B, Kesman-Leegte IGAT, Postma DS, Timens W. Effect of 1 year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J.* 2005; 26:835–845. [PubMed: 16264044]
- Yang G, Kong L, Zhao W, et al. Emergence of chronic non-communicable diseases in China. *Lancet.* 2008; 372:1697–1705. [PubMed: 18930526]

Highlights

Cigarette smoke extract (CSE) exposure did not increase ROS production in AT I cells.

CSE exposure increased H₂O₂ levels in endothelial cell (MVECL) cultures.

When in co-culture with AT I cells, CSE-induced H₂O₂ production by MVECL was reduced.

Total antioxidant capacity in co-cultures may explain reduction in H₂O₂ levels.

Figure 1. H₂O₂ production in single and co-cultures of neonatal, young and old MVECLs and AT I cells with and without exposure to CSE

Single and co-cultures of MVECLs and age-matched AT I cells were exposed to CSE (1, 10, 50 and 100%) or vehicle control for 24 hours and assayed for extracellular H₂O₂ production. **A)** MVECLs grown as single cultures show a dose dependent increase in H₂O₂ ($p < 0.001$), and a significant effect based on age ($p < 0.05$) with young cells differing from the other two groups ($p < 0.05$). There was an interaction effect between age and CSE concentration ($p < 0.001$). **B)** AT I cells grown as single cultures did not show a change in H₂O₂ nor was there an effect related to age or an interaction between age and CSE concentration. **C)** Co-cultures of age-matched MVECLs and AT I cells exposed to CSE or vehicle control show up to a 50% reduction in H₂O₂, compared to single cultures. There was not an effect related to CSE concentration, however there was an effect related to age ($p < 0.05$) with young cells producing less H₂O₂. There was no interaction effect between age and CSE concentration. Error bars $\pm$ S.E. ($n=3$). (* denotes the group that differs, # denotes statistical difference between groups, $p < 0.05$).

Figure 2. Intracellular ROS production in single cultures of neonatal, young and old MVECLs and AT I cells with and without exposure to CSE

Single and co-cultures of MVECLs and age-matched AT I cells were exposed to CSE (1, 10, 50 and 100%) or vehicle control for 24 hours and assayed for intracellular ROS production. **A)** MVECLs grown as single cultures did not show an effect related to CSE. There was an effect related to age ($p < 0.05$) with young cells producing less intracellular ROS compared to neonatal or old cells. There was not an interaction effect between age and CSE concentration. **B)** AT I cells grown as single cultures did not show an effect related to CSE concentration. There was an effect related to age ($p < 0.05$) with less intracellular ROS production by neonatal AT I cells compared to young and old. There was not an interaction effect between age and CSE concentration. Error bars $\pm$ S.E., ($n=3$). (* denotes the group that differs, # denotes statistical difference between groups, $p < 0.05$).

Figure 3. NO, ONOO⁻, and OH⁻ production in neonatal, young, and old MVECLs grown in co-culture with age-matched AT I cells and exposed to CSE

Monolayers of MVECLs were exposed to CSE (1, 10, 50 and 100%) or vehicle control for 24 hours and assayed for NO, ONOO⁻ and OH⁻ production. **A)** MVECLs show an increase in NO production following 24 hours exposure to CSE (1, 10, 50 and 100%) ($p < 0.01$).

There was neither an effect related to age nor an interaction between age and CSE concentration. **B)** MVECLs show an increase in the production of ONOO⁻ and OH⁻ in response to CSE ($p < 0.001$). There was neither an effect related to age nor an interaction effect between age and CSE concentration. Error bars $\pm$ S.E, (n=3).

Figure 4. Total antioxidant capacity of single and co-cultures of neonatal, young and old MVECLs and AT I cells with and without exposure to CSE

Single and co-cultures of MVECLs and age-matched AT I cells were exposed to CSE (1, 10, 50 and 100%) or vehicle control for 24 hours and the cell supernatant assayed for total antioxidant capacity. **A)** The total antioxidant capacity of cell supernatants from cultures of neonatal, young and old MVECLs with exposure to CSE (1, 10, 50 and 100%) or vehicle control. There was an effect related to CSE concentration ($p < 0.001$) and age ($p < 0.001$) with neonatal cells differing ($p < 0.05$) from the other two age groups. There was an interaction effect between age and CSE concentration ($p < 0.001$). **B)** The total antioxidant capacity of cell supernatants from cultures of neonatal, young and old AT I cells with exposure to CSE or vehicle control. There was an effect related to CSE ($p < 0.001$) and age ($p < 0.05$) with young AT I cells differing ($p < 0.05$) from neonatal and old AT I cells. There was an interaction effect between age and CSE concentration ($p < 0.05$). **C)** The total antioxidant capacity of cell supernatants from co-cultures of neonatal, young and old MVECLs and age-matched AT I cells. There was an effect related to CSE ($p < 0.001$) and age ($p < 0.001$) with the antioxidant capacity of young cells differing ($p < 0.05$) from the other two groups. There was an interaction effect between age and CSE concentration ($p < 0.001$). Error bars $\pm$ S.E. (n=3). (*) denotes the group that differs, # denotes statistical difference between groups, $p < 0.05$).

Table 1

Genes that demonstrate a significant change when MVECLs were grown as a co-culture.

The oxidant and antioxidant defense qPCR pathway gene array was used to determine the gene expression profile of MVECLs grown as single cultures and as co-cultures with age-matched AT I cells with 10 % CSE exposure. Eight genes were significantly up-regulated or down-regulated in 10% CSE exposed MVECLs grown as co-culture compared to MVECLs grown as a single culture.

Gene Name	Abbreviation	Role of Gene in Oxidative Stress	Fold Change [†]	P Value
Apolipoprotein e	Apo e	Exact mechanism unknown; found to have antioxidant effect in the lung.	-2.25	0.022
Cytoglobin	Cygb	Oxygen transporter protein; facilitates the diffusion of oxygen and scavenges NO or ROS	-3.42	0.024
Excision repair cross complementing rodent repair deficiency	Ercc6	Participates in the repair of damaged DNA	+3.18	0.045
NAD(P)H:quinone oxidoreductase 1	Nqo1	Performs two-electron reduction of quinones to hydroquinones, reducing ROS production	+2.44	0.040
Prion protein	Prnp	Exact mechanism unknown; found to play a role in apoptosis	-8.68	0.010
Solute carrier family 38, member 1	Slc38a1	Involved in the transport of amino acids, a process that may generate ROS	-17.26	0.022
Tropomodulin 1	Tmod1	Exact mechanism unknown; possesses reported peroxidase activity	-5.71	0.048
Zinc finger, MYND-type containing 17	Zmynd17	Stabilizes proteins and typically functions as an interaction molecule that binds DNA, RNA proteins or small molecules; may serve to transduce oxidative stress signal	+9.98	0.008

(n=3;

[†] co-culture/single culture).