Age-specific effects on rat lung glutathione and antioxidant enzymes after inhaling ultrafine soot

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**ONLINE DATA SUPPLEMENT**

**Online Supplementary Material and Methods**

*Flame and particle generation:* Premixed flame particles (PFP) were generated using a coannular premixed flame burner as previously detailed (1, 2). Briefly, the burner was fed a mixture of ethylene, oxygen and argon to generate the flame. Filtered dried air was added to the flow downstream and effluent passed through a heated 3-way catalyst to remove $NO_x$ and CO. PFPs were diluted with clean HEPA and CBR (chemical/biological/radiology) filtered air and mixed before entering the inhalation exposure chamber. The particle mass concentration in the chamber was 22.4 ± 5.6 μg/$m^3$ PFP (mean ± SD) based on gravimetric filter measurement. PFP particle mobility diameter was 70.6 nm ± 1.5 (geometric mean ± geometric SD), determined using a scanning mobility particle sizer (SMPS). Particle numbers were 9.37 x $10^4$ ± 4.8 x $10^3$, as determined used a condensation particle counter. Particles were high in organic carbon and had an EC:OC ratio of 0.58. The total amount of polycyclic aromatic hydrocarbons were 54 ng/$m^3$ attached to PFP, and 405 ng/$m^3$ in the gas phase. For HOOH rate determinations, PFP were collected on 0.22 μm Teflon filters (Zefluor, PALL corporation) and stored in the dark at -20 ˚C prior to use.

*Animal exposure protocol:* Eight-week old reproductively capable young male adults and newborn postnatal male Sprague Dawley rats with accompanying dams were obtained from Harlan Laboratories. Animals were allowed to acclimate in CBR filtered air (FA) until newborns reached 7 days of age. Adult rats were provided with Laboratory Rodent Diet (Purina Mills, St. Louis, MO) and water *ad libitum*. Animals were exposed to 22.4 ± 5.6 μg/$m^3$ PFP atmosphere or FA for 6 hours in two exposure chambers previously described (1, 2). All animal experiments were performed under protocols approved by the University of California Davis IACUC in

accordance with National Institutes of Health guidelines. Animals were necropsied at 2, 24 and 48 hours, designated as PFP2, PFP24, and PFP48 groups, respectively, following cessation of the 6 hour exposure. All animals were euthanized by an intraperitoneal injection of an overdose of pentobarbital (150 mg/kg).

*High Performance Liquid Chromatography (HPLC):* Lungs were inflated with a warmed 37°C solution of 1% agarose (Sigma Chemical, St. Louis, MO) in sulfur deficient Waymouths MB 752/1 media (Life Technologies, Grand Island, NY) and subsequently placed in ice cold Ham's F-12 media (Life Technologies, Grand Island, NY) for at least 15 minutes to solidify agarose solution in preparation for dissection. Airways and surrounding parenchyma were microdissected and homogenized in a solution containing 200mM methanesulfonic acid and 5mM diethylenetriaminepentaacetic acid (3). Samples were centrifuged and both supernatant and pellet were collected. Sample glutathione (GSH) and glutathione disulfide (GSSG) concentrations were determined through integration of peak areas as previously described (4), with the following alterations. A 10 to 20 µl sample of supernatant was injected into a reverse phase HPLC system connected to a Waters 5040 analytical cell electrochemical detector (Waters, Wilford, MA) measured against a known GSH and GSSG standard curve and retention time standards. Responses were linear from 2 to 500 ng of GSH ($R^2$ = 0.9949) and 4 to 500 ng of GSSG ($R^2$ = 0.9994). Values detected below these limits were treated as nondetects. Pellets were dissolved in 1M NaOH at 60°C overnight and total protein concentrations were determined using the micro Lowry assay (Sigma Chemical, St. Louis, MO)

*RNA isolation and Real time PCR (RT-PCR):* Lung compartmental RNA was isolated from microdissected intrapulmonary airways and surrounding parenchymal tissue from RNAlater

(Ambion, Austin, TX) stabilized lung tissue using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (5). RNA purity was confirmed through spectrophotometric absorbance at 260/280 nm. Quantification of GCLc, GCLm, GPX1, GSTM1, GSTP1, GSTT1, GSR and HPRT gene expression in the airway and parenchymal compartments were performed using inventoried Taqman probes and primers (Applied Biosystems, Foster City, CA) as previously described (5, 6). A complete list of genes with Applied Biosystems assay IDs, gene names and NCBI RefSeq accession numbers are presented in Table 1. Results were calculated using the comparative Ct method (7, 8) using Hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the reference gene. HPRT was chosen as the reference gene due to consistency and low variance between exposure groups as previously assessed (1, 9). Results are expressed as a fold change in gene expression relative to filtered animal controls at the 24 hour time point of the same age, unless otherwise stated.

*Western Blotting:* Flash-frozen lung tissue was homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and protein concentrations determined with the Bradford assay (Bio-Rad, Hercules, CA). Samples were reduced for SDS-PAGE and 20-40 µg protein/lane were electrophoresed on 12% Bis-Tris polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The optimal amount of protein loaded was determined through a series of dilutions to optimize for quantification linearity. To reduce nonspecific antibody binding, membranes were incubated in 5% albumin and each of the following antibodies at manufacturer recommended concentrations: rabbit anti-GSR (Abcam, Cambridge, MA) at 1:1000, rabbit anti-GCL (Neomarkers, Fremont, CA) at 1:1000, rabbit anti-GPX1 (Abcam, Cambridge, MA) at 1:1000 and rabbit anti-actin (Abcam, Cambridge, MA) at 1:1000 overnight. Protein bands were amplified using a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary at 1:2500 (Abcam, Cambridge, MA) and

visualized using Amersham ECL reagents (GE Healthcare, Piscataway, NJ) on Amersham ECL Hyperfilm (GE Healthcare). Film was scanned on an Epson Precision 1640SU scanner (Epson America, Long Beach, CA) and protein bands quantified using ImageJ (NIH, Bethesda, MD). Results are expressed as relative abundance in protein expression relative to filtered air animals of the same age.

*Immunohistochemistry:* Lungs were inflated with 37% formaldehyde vapor bubbled under 30 cm hydrostatic pressure for 1 hour as previously described (10, 11). Samples were stored in 1% paraformaldehyde for less than 24 hours prior to tissue processing and paraffin embedment. Paraffin sections on poly-L-lysine coated slides from all groups were stained simultaneously to minimized variability between runs and were immunostained for rabbit anti-GSR (Abcam, Cambridge, MA) at 1:500, rabbit anti-GPX1 (Abcam, Cambridge, MA) at 1:2000 and rabbit anti-GCL (Neomarkers, Fremont, CA) at 1:300. The concentration of primary antibody was determined through a series of dilutions to optimize for staining density while minimizing background. This procedure was performed according to previously described methods (9, 12) with several alterations. Following tissue hydration, endogenous peroxidase activity was quenched with a 10% solution of hydrogen peroxide. To eliminate nonspecific primary antibody binding, tissue sections were blocked with 5% albumin. Primary antibodies were allowed to incubate in a humidified chamber overnight at 4°C. Signal was amplified using the Vectastain IgG Avidin-Biotin-HRP Kit (Vector Labs, Burlingame, CA), and visualized using nickel chloride enhanced 3,3'-diaminobenzidine tetrachloride (Sigma Chemical, St. Louis, MO) as the chromagen. Controls included substitution of primary antibody with phosphate-buffered saline to ensure specific positive staining.

*HOOH Assay:* The rate of HOOH production from 18.4 µg of PFP collected on Teflon filters was measured in triplicate in a cell-free surrogate lung fluid (SLF) designed to mimic endogenous lung fluid (13). The SLF contained 0.01 M phosphate buffer (pH 7.3) and 0.114 M NaCl and was treated with chelex 100 resin (Biorad) to remove trace metal contamination (13). Prior to PFP addition, the following antioxidants were added to the SLF: L-ascorbate (to achieve an SLF concentration of 0.20 mM), citrate (0.30 mM), glutathione (0.10 mM) and sodium urate (0.10 mM). We also added a final concentration of 18.4 mM of 2,2,2-trifluoroethanol (TFE) as a filter wetting agent to allow the SLF to extract the PFP from the hydrophobic Teflon filter substrates. Filter sections with a known PFP mass were then added to the SLF-antioxidant-TFE mixture. All reactions were carried out at room temperature in acid-washed Teflon vials in the dark. Samples were mixed continuously on an orbital shake table (VWR OS-500) to ensure complete mixing and enhance particle extraction from the filters. HOOH production was measured at 0.0, 0.5, 1.0, and 1.5 hours using HPLC with fluorescence detection. Quantification of HOOH is described in detail elsewhere (14); an identical instrument and set-up to (15) was used in this work. The rate of HOOH production was calculated by taking the slope of the linear range of HOOH concentration versus time. Duplicate filter blanks consisting of an identically sized filter punch with TFE but without PFP were also analyzed to identify background HOOH production. The rate of HOOH production from filter blanks were identical to the solution blank (without filter or TFE), indicating the filter and TFE did not produce a measurable amount of HOOH.

The rate of HOOH production from ambient $PM_{2.5}$ collected in Fresno, CA during the summer of 2008 was measured in duplicate as a comparison. A detailed description of Fresno $PM_{2.5}$ sampling can be found in (15). Fresno $PM_{2.5}$ samples were treated in an identical manner to PFP and filter blanks, using the same-sized filter section. The mass normalized rate of HOOH production from PFP was similar to that of ambient Fresno $PM_{2.5}$ (Figure E1).

*Statistics:* All data are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical outliers were eliminated using the extreme studentized deviate method (Graphpad, La Jolla, CA). Undetected and samples observed below detection limit were treated as nondetects, and values were imputed using the natural-log regression on order statistics (lnROS) method (16, 17) using ProUCL (U.S. EPA, Atlanta, GA). Multivariate analysis of variance (MANOVA) was applied against age, compartment and exposure factors when appropriate. Multiple comparisons for factors containing more than two levels were performed using Fisher's Protected Least Significant Difference (PLSD) method. Pair-wise comparisons were performed individually using a one-way ANOVA followed by PLSD post hoc analysis using StatView (SAS, Cary, NC). *P* values of $< 0.05$ were considered statistically significant.

## References


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Figure E1. The blank-corrected rate of HOOH production from PFP and from ambient $PM_{2.5}$ collected in Fresno, CA during the summer of 2008. Error bars are the standard deviation of triplicate measurements.

Table E1: GCLc and GCLm gene expression significance values

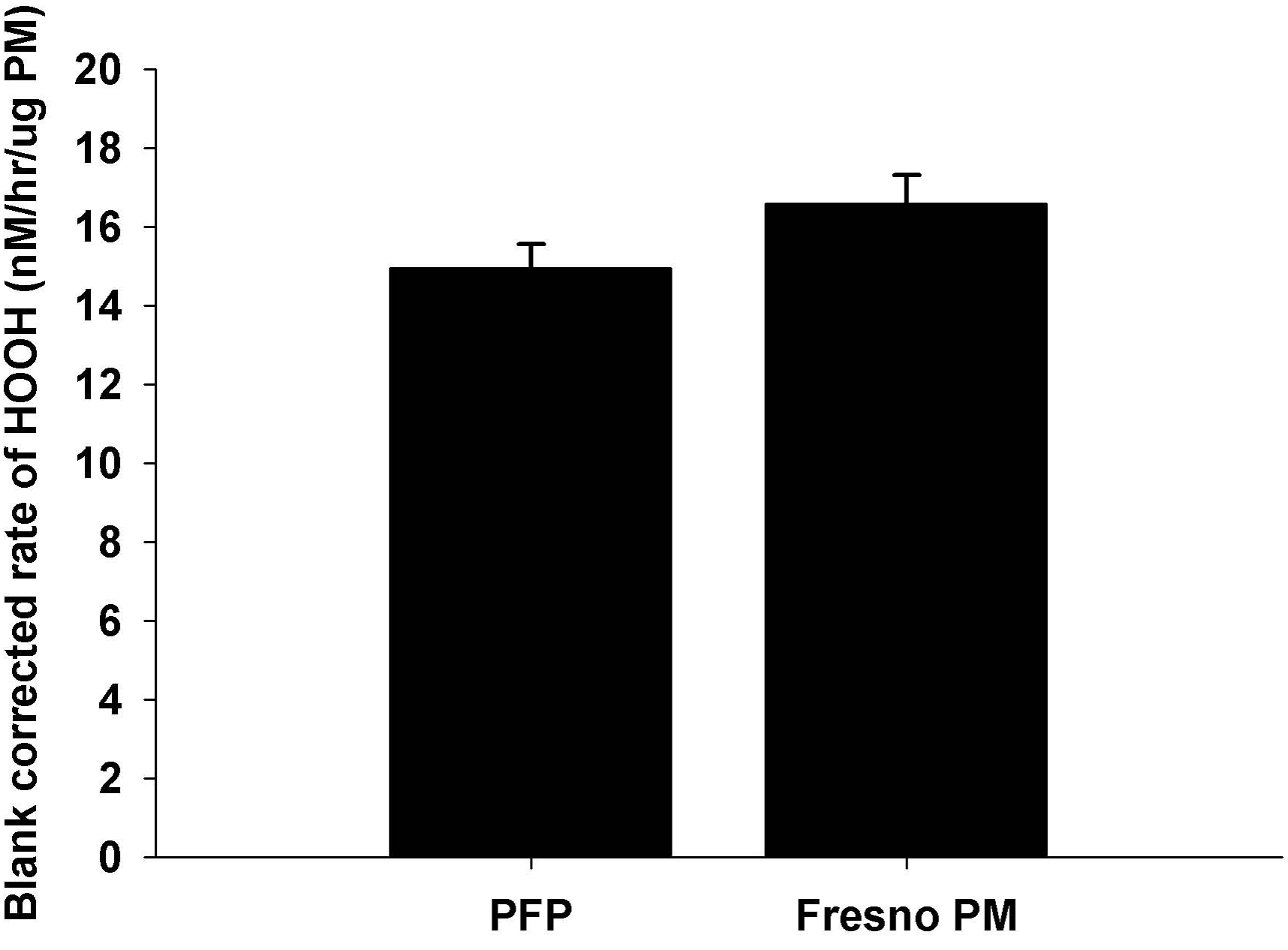
| | | | GCLc | | | | GCLm | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Adult | | Neonate | | Adult | | Neonate | |
| | | | Airway | Parenchyma | Airway | Parenchyma | Airway | Parenchyma | Airway | Parenchyma |
| GCLc | Adult | Airway | | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** |
| | | Parenchyma | | | 0.8004 | **0.0026 ↓** | **0.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** |
| | Neonate | Airway | | | | **0.0013 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** |
| | | Parenchyma | | | | | 0.2796 | 0.0813 | 0.0829 | **0.0431 ↓** |
| GCLm | Adult | Airway | | | | | | 0.4929 | 0.4987 | 0.3268 |
| | | Parenchyma | | | | | | | 0.9926 | 0.7652 |
| | Neonate | Airway | | | | | | | | 0.7581 |
| | | Parenchyma | | | | | | | | |

Table E1. P-values from pair-wise comparisons against GCLc and GCLm basal gene expression in adult and neonatal airway and parenchymal compartments. Significant comparisons are bolded with an up or down arrow indicating significant up or downregulation, respectively

Table E2: GST isoforms gene expression significance values

| | | | Adult | | | | | | Neonates | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Airway | | | Parenchyma | | | Airway | | | Parenchyma | | |
| | | | GSTM | GSTP | GSTT | GSTM | GSTP | GSTT | GSTM | GSTP | GSTT | GSTM | GSTP | GSTT |
| Adult | Airway | GSTM | | **<.0001 ↓** | **<.0001↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** | **<.0001 ↓** |
| | | GSTP | | | **<.0001 ↓** | **<.0001 ↓** | 0.2748 | **<.0001 ↓** | **0.002 ↓** | **0.0208 ↓** | **<.0001↓** | **<.0001 ↓** | 0.1707 | **<.0001 ↓** |
| | | GSTT | | | | 0.1219 | **<.0001 ↑** | 0.8163 | **0.0189 ↑** | **0.0018 ↑** | 0.672 | 0.6467 | **<.0001 ↑** | 0.4813 |
| | Parenchyma | GSTM | | | | | **<.0001 ↑** | 0.0765 | 0.3623 | 0.0795 | 0.0595 | **0.0469 ↓** | **0.0042 ↑** | **0.0264 ↓** |
| | | GSTP | | | | | | **<.0001 ↓** | **<.0001 ↓** | **0.0011 ↓** | **<.0001 ↓** | **<.0001 ↓** | **0.0157 ↓** | **<.0001 ↓** |
| | | GSTT | | | | | | | **0.0107 ↑** | **0.0009 ↑** | 0.8399 | 0.8209 | **<.0001 ↑** | 0.6364 |
| Neonates | Airway | GSTM | | | | | | | | 0.4098 | **0.0086↑** | **0.006 ↓** | 0.0595 | **0.0031 ↓** |
| | | GSTP | | | | | | | | | **0.0008 ↓** | **0.0005 ↓** | 0.2957 | **0.0002 ↓** |
| | | GSTT | | | | | | | | | | 0.989 | **<.0001 ↑** | 0.8033 |
| | Parenchyma | GSTM | | | | | | | | | | | **<.0001 ↑** | 0.8051 |
| | | GSTP | | | | | | | | | | | | **<.0001 ↓** |
| | | GSTT | | | | | | | | | | | | |

Table E2. P-values from pair-wise comparisons against Glutathione S-transferase isoforms Mu, Pi and Theta genes basally expressed in adult and neonatal airway and parenchymal compartments. Significant comparisons are bolded with an up or down arrow indicating significant up or downregulation, respectively


Blank corrected rate of HOOH (nM/hr/ug PM)
20
18
16
14
12
10
8
6
4
2
0
PFP
Fresno PM