Supplemental Material for Environmental Health Perspectives

Mitochondria-Derived Reactive Intermediate Species Mediate Asbestos-induced Genotoxicity and Oxidative Stress-Responsive Signaling Pathways

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Toxicity of asbestos

The cellular toxicity of asbestos was measured by determining the survival fraction with the CyQuant cell proliferation Assay kit (Invitrogen). Briefly, exponentially growing cells in a 96-well tissue culture plate were treated with graded doses of asbestos fibers (0.5, 1, 2, and 4 µg/cm²) for 72 hours; then culture media were removed and the cellular nuclei acids content of each control and treated group was measured with a fluorescence excitation of 480nm using a Synergy 2 multi-detection Microplate Reader (BioTek Instruments, Inc, Vermont). The surviving fractions (percentage over untreated controls) for treated groups were calculated.

Real-time Quantitative PCR

The mtDNA copy number was determined by real-time SYBR Green PCR using the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems). Genomic DNA was isolated and concentration was measured by spectrophotometry. For each sample, we amplified a 189-bp fragment of the nuclear encoded 18S rRNA gene and a 172-bp fragment of the mtDNA encoded 12S rRNA gene. The primer sequences are as follows: 18S sense, 5’-GGAGTATGTTGCAAGGCTG-3’; 18S antisense, 5’-CGCTCAACTAAAGACG-3’; 12S sense, 5’-AGAACACTACGACCACAGC-3’; and 12S antisense, 5’-ACTGCGCTTACTTTGGAGC-3’. All reactions were done in triplicate. The PCR conditions were: 95°C for 15 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Relative quantification of mtDNA reported as mtDNA/nDNA ratio was done with the comparative threshold cycle (C_T) method as described previously (Partridge et al. 2007; Shao et al. 2006).

Mitochondrial membrane potential

JC-1, a membrane potential-sensitive fluorescent probe (Invitrogen), was used to determine the mitochondrial membrane potential of ρ₀ SAE cells (Liu et al. 2005; Reers et al. 1995). Exponentially growing parental and ρ₀ SAE cells were treated with 10 µmol/L JC-1 in growth medium for 30 min at 37°C. Extra dye was removed by washing with warm PBS and cells were maintained in regular medium on a heated 37°C stage. Cells were immediately visualized and images were captured on a Nikon laser confocal microscope (Nikon eclipse TE2000-U; excitation 488 nm and 543 nm for green and orange fluorescence, respectively).

Oxygen consumption

The oxygen consumption rate in live cells was measured as described previously (King et al. 1992; Partridge et al. 2007). Cells were counted and a minimum of 5 x 10⁶ cells were resuspended in 1.5 mL of Opti-MEM (glucose free and sodium pyruvate supplemented
medium). Oxygen concentration was monitored over 3 min and recorded for every 10 seconds at 37°C in a Hansatech (MA) Clark's oxygen electrode unit.

**Cytochrome c Oxidase (COX) and succinate dehydrogenase (SDH) activity**

COX and SDH activities were measured with biochemical assays as described (King et al. 1967; Partridge et al. 2007; Salviati et al. 2002). COX activity was indicated by the capability of the cell lysates to oxidize reduced cytochrome c at 550 nm (nmol oxidized cytochrome c per min per ml cell lysate) as measured by spectrophotometry. SDH activity was assessed by measuring the oxidation of succinate coupled to the reduction of DCPIP (2,6-dichlorophenolindophenol, an electron acceptor that is blue when oxidized and colorless when reduced) by the SDH reaction at 600 nm with spectrophotometry. The enzyme activity was indicated by nmol oxidized succinate per min per ml cell lysate. Both COX and SDH activities were normalized to mg of protein in the cell lysate.

**Intracellular superoxide measurement with dihydroethidium (DHE)**

Intracellular superoxide was determined using the fluorescent probe DHE (Invitrogen) as described previously (Zhou et al. 2008). Exponentially growing ρ0 or parental SAE cells were stained with 2µmol/L DHE in regular medium for 45 min at 37°C. Cells were then trypsinized and suspended in PBS. The DHE fluorescence was measured by flow cytometry in the FL3 channel on a FACSCalibur (Becton Dickinson).
Supplemental Material, Table 1. Real-time PCR screening of mtDNA content in EtBr-treated SAE cells and verification of the $\rho^0$ status.

| Samples                              | mtDNA/nDNA* Ratio  |
|--------------------------------------|---------------------|
| SAE Cells                            | 1.0000 ± 0.0446     |
| 23 days                              | 0.0100 ± 0.0037     |
| 29 days                              | 0.0059 ± 0.0013     |
| EtBr-treated SAE cells               |                     |
| 37 days                              | 0.0039 ± 0.0005     |
| 55 days                              | 0.0027 ± 0.0005     |
| 63 days                              | 0.0022 ± 0.0008     |
| $\rho^0$ SAE cells +1 passage        | 0.0059 ± 0.0004     |
| $\rho^0$ SAE cells +3 passage        | 0.0097 ± 0.0024     |
| $\rho^0$ SAE cells +5 passage        | 0.0165 ± 0.0019     |
| $\rho^0$ SAE cells +7 passage        | 0.0779 ± 0.0275     |
| $\rho^+$ primary human skin Fibroblast | 1.0000 ± 0.1877   |
| $\rho^0$ primary human skin Fibroblast | 0.0046 ± 0.0007   |

* nDNA = nuclear DNA

**Note:** mitochondrial DNA contents relative to nuclear DNA contents was determined using quantitative real-time PCR. The resultant mtDNA/ n DNA ratios in EtBr-treated samples were normalized to the ratio in untreated SAE cells.
Supplemental Material, Table 2. A list of all the genes examined.

| Gene   | Gene   | Gene   | Gene   | Gene   | Gene   |
|--------|--------|--------|--------|--------|--------|
| A2M    | CCR5   | FN1    | IL2    | MAPK3  | PTGER2 |
| ACE    | CCR7   | GNLY   | IL2RA  | MAPK8  | PTGER3 |
| ADRB1  | CD19   | GUSB   | IL2RB  | MC2R   | PTGFR  |
| ADRB2  | CD28   | GZMB   | IL2RG  | MYH6   | PTGIR  |
| AGTR1  | CD34   | HLA-DRA| IL3    | NFKB1  | PTGIS  |
| AGTR2  | CD38   | HLA-DRB1| IL4   | NFKB2  | PTGS1  |
| ALOX12 | CD3E   | HMOX1  | IL5    | NOS2A  | PTGS2  |
| ALOX5  | CD4    | HPGD   | IL6    | NR3C1  | PTPRC  |
| ANXA1  | CD40   | HRH1   | IL7    | PDE4A  | REN    |
| ANXA3  | CD40LG | HRH2   | IL8    | PDE4B  | RPL3L  |
| ANXA5  | CD68   | HRH3   | IL9    | PDE4C  | SELE   |
| B2M    | CD80   | HTR3A  | ITGAL  | PDE4D  | SELP   |
| BAX    | CD86   | HTR3B  | ITGAM  | PGK1   | SKI    |
| BCL2   | CD8A   | ICAM1  | ITGB1  | PLA2G10| SMAD3  |
| BCL2L1 | CES1   | ICOS   | ITGB2  | PLA2G1B| SMAD7  |
| BDKRB1 | COL4A5 | IFNG   | KLK1   | PLA2G2A| STAT3  |
| BDKRB2 | CSF1   | IKBKB  | KLK14  | PLA2G2D| TBX21  |
| C3     | CSF2   | IL10   | KLK15  | PLA2G4C| TBX2A2R|
| CACNA1C| CSF3   | IL12A  | KLK2   | PLA2G5 | TBXAS1 |
| CACNA1D| CTLA4  | IL12B  | KLK3   | PLA2G7 | TFRC   |
| CACNA2D1| CXCL10| IL13   | KLKB1  | PLCB2  | TGFβ1  |
| CACNB2 | CXCL11 | IL15   | KNG1   | PLCB3  | TNF    |
| CACNB4 | CXCR3  | IL17   | LRP2   | PLCB4  | TNFRSF18|
| CASP1  | CYP1A2 | IL18   | LTA    | PLCD1  | TNFRSF1A|
| CCL19  | CYP7A1 | IL1A   | LTA4H  | PLCE1  | TNFRSF1B|
| CCL2   | CYSLTR1| IL1B   | LT4B4R | PCLG1  | TNFSF13B|
| CCL3   | ECE1   | IL1R1  | LT4B4R2| PCLG2  | VCAM1  |
| CCL5   | EDN1   | IL1R2  | LTC4S  | PRF1   | VEGF   |
| CCR2   | FAS    | IL1RAPL2| MAPK1 | PTAFR  |        |
| CCR4   | FASLG  | IL1RL1 | MAPK14 | PTGDR  |        |

Note: 8 genes from the pre-designed immune and inflammation arrays overlapped with each other. 178 genes are in list, excluding 3 housekeeping genes in each array.
Supplemental Material, Table 3. Differentially expressed genes in asbestos-treated SAE cells.

| Genes   | RQ (Asb vs. Ctrl*) | P.Value | Genes   | RQ (Asb vs. Ctrl) | P.Value |
|---------|--------------------|---------|---------|------------------|---------|
| 12 hr   |                    |         | 48 hr   |                  |         |
| ADRB2   | 0.85               | 0.032   | ADRB1   | 2.13             | 0.01    |
| AGTR1   | 0.61               | 0.024   | ANXA5   | 1.15             | 0.008   |
| CES1    | 1.67               | 0.012   | BAX     | 1.69             | 0.038   |
| CSF2    | 11.47              | 0.049   | BCL2    | 2.38             | 0.013   |
| EDN1    | 0.53               | 0.033   | CACNB4  | 1.37             | 0.004   |
| HMOX1   | 2.73               | 0.007   | CCL2    | 3.33             | 0.027   |
| HRH1    | 1.53               | 0.021   | CCR4    | 10.82            | 0.019   |
| ICAM1   | 5.61               | 0.037   | CD4     | 0.54             | 0.008   |
| LTA     | 2.16               | 0.037   | CSF1    | 2.35             | 0.032   |
| PTAFR   | 0.74               | 0.002   | CSF2    | 702.65           | 0.008   |
| RPL3L   | 42.57              | 0.008   | CSF3    | 92.48            | 0.019   |
| TBXAS1  | 1.53               | 0.034   | CYSLTR1 | 0.32             | 0.001   |
| TFRC    | 0.77               | 0.044   | FAS     | 2.21             | 0.034   |
| VEGF    | 1.44               | 0.003   | FN1     | 1.41             | 0.014   |
| 24 hr   |                    |         |         |                  |         |
| ANXA3   | 0.55               | 0.033   | HLA-DRA | 3.00             | 0.009   |
| BAX     | 1.22               | 0.001   | HMOX1   | 3.46             | 0.014   |
| CACNB4  | 1.42               | 0.025   | HRH1    | 3.12             | 0.017   |
| CCR7    | 35.71              | 0.00002 | ICAM1   | 13.53            | 0.007   |
| CD34    | 0.04               | 0.006   | IL1A    | 23.60            | 0.049   |
| CD40    | 1.23               | 0.043   | IL6     | 14.96            | 0.013   |
| CSF1    | 1.66               | 0.008   | IL8     | 8.69             | 0.008   |
| CSF2    | 29.70              | 0.007   | LTA     | 7.93             | 0.018   |
| ECE1    | 1.21               | 0.006   | NFKB2   | 3.19             | 0.014   |
| EDN1    | 0.46               | 0.012   | PLCD1   | 0.70             | 0.027   |
| HMOX1   | 4.01               | 0.006   | PLCE1   | 0.67             | 0.017   |
| HRH1    | 1.87               | 0.032   | PTGFR   | 1.71             | 0.037   |
| HRH2    | 1.95               | 0.028   | SELE    | 24.53            | 0.003   |
| ICAM1   | 9.34               | 0.006   | TBXAS2R | 0.52             | 0.047   |
| IL1A    | 9.71               | 0.027   | TFRC    | 1.61             | 0.008   |
| IL6     | 4.97               | 0.03    | TNF     | 130.57           | 0.001   |
| NFKB2   | 1.68               | 0.049   | TNFRSF1B| 1.77             | 0.025   |
| PTAFR   | 1.59               | 0.045   | VEGF    | 3.16             | 0.013   |
| PTGER2  | 0.91               | 0.044   |         |                  |         |
| PTGFR   | 1.89               | 0.032   |         |                  |         |
| PTGIR   | 1.80               | 0.0004  |         |                  |         |
| PTGS2   | 7.33               | 0.002   |         |                  |         |
| TBXAS1  | 1.71               | 0.026   |         |                  |         |
| TGFB1   | 1.58               | 0.028   |         |                  |         |
| TNF     | 96.79              | 0.006   |         |                  |         |
| VEGF    | 2.30               | 0.006   |         |                  |         |

*Asb = Asbestos, Ctrl = Control; †Paired T-test by Statminer

The RQ value of each gene is plotted in Main text, Figure 4B, left.
Supplemental Material, Table 4. Differentially expressed genes in asbestos-treated $\rho^0$ SAE cells.

|         | Genes        | RQ (Asb vs. Ctrl*) | P.Value† | Genes      | RQ (Asb vs. Ctrl) | P.Value† |
|---------|--------------|--------------------|----------|------------|------------------|----------|
| 12 hr   | $CSF1$       | 1.17               | 0.01     | $AHRB2$    | 0.70             | 0.017    |
|         | $CSF2$       | 1.91               | 0.005    | $CACNA2D1$ | 0.80             | 0.028    |
|         | $EDN1$       | 0.60               | 0.047    | $CCR4$     | 3.24             | 0.039    |
|         | $FAS$        | 0.76               | 0.016    | $CD68$     | 1.38             | 0.03     |
|         | $IL10$       | 13.79              | 0.004    | $CSF3$     | 1.64             | 0.044    |
|         | $IL1A$       | 0.83               | 0.024    | $EDN1$     | 0.50             | 0.042    |
|         | $PLCE1$      | 0.90               | 0.015    | $HMOX1$    | 1.60             | 0.031    |
|         | $PTGER2$     | 0.85               | 0.012    | $HRH1$     | 1.43             | 0.039    |
| 24 hr   | $ANXA3$      | 0.74               | 0.0001   | $ITGB2$    | 0.74             | 0.018    |
|         | $B2M$        | 0.81               | 0.025    | $MAPK1$    | 0.75             | 0.018    |
|         | $CACNB4$     | 1.59               | 0.018    | $MC2R$     | 5.66             | 0.042    |
|         | $CSF1$       | 1.32               | 0.038    | $PLCE1$    | 0.64             | 0.044    |
|         | $HMOX1$      | 1.50               | 0.012    |            |                  |          |
|         | $PLCB4$      | 0.71               | 0.014    |            |                  |          |
|         | $PLCG1$      | 0.87               | 0.046    |            |                  |          |
|         | $PTGER2$     | 0.76               | 0.036    |            |                  |          |
|         | $PTGIS$      | 0.45               | 0.003    |            |                  |          |
|         | $TFRC$       | 0.85               | 0.008    |            |                  |          |
|         | $TNFRSF1B$   | 0.77               | 0.024    |            |                  |          |

*Asb = Asbestos, Ctrl = Control; †Paired T-test by StatMiner

The $\log_{10}$RQ value of each gene is plotted in Main text, Figure 4B, right.
Supplemental Material, Table 5. Expressed genes at baseline in $\rho^0$ versus parental SAE cells.

| Genes     | RQ ($\rho^0$ vs. parental SAE cells) | P.Value* | Genes     | RQ ($\rho^0$ vs. parental SAE cells) | P.Value* |
|-----------|-------------------------------------|----------|-----------|-------------------------------------|----------|
| ACE       | 0.1                                 | 0.006    | IL1B      | 100.5                               | 0.037    |
| ADRB1     | 3.4                                 | 0.003    | IL1RAPL2  | 2.8                                 | 0.006    |
| ANXA3     | 8.3                                 | 0.004    | IL6       | 3.4                                 | 0.005    |
| ANXA5     | 0.5                                 | 0.041    | IL7       | 1.7                                 | 0.038    |
| C3        | 3.2                                 | 0.009    | IL8       | 0.4                                 | 0.032    |
| CACNA2D1  | 7.6                                 | 0.047    | ITGB2     | 0.5                                 | 0.02     |
| CCL2      | 0.2                                 | 0.017    | KLKB1     | 0.3                                 | 0.032    |
| CCL3      | 282.2                               | 0.006    | MAPK1     | 0.6                                 | 0.004    |
| CCL5      | 46.8                                | 0.003    | MC2R      | 0.6                                 | 0.031    |
| CD40      | 0.5                                 | 0.009    | NFKB1     | 0.6                                 | 0.044    |
| CES1      | 13.1                                | 0.013    | PDE4A     | 2.8                                 | 0.013    |
| COL4A5    | 0.3                                 | 0.011    | PLA2G7    | 0.1                                 | 0.041    |
| CSF1      | 1.5                                 | 0.003    | PLCD1     | 0.7                                 | 0.021    |
| CSF2      | 2.5                                 | 0.042    | PLCE1     | 0.4                                 | 0.009    |
| CSF3      | 17.5                                | 0.006    | PLCG2     | 472.1                               | 0.01     |
| CXCL10    | 85.7                                | 0.005    | PTGIR     | 6.4                                 | 0.043    |
| EDN1      | 0.3                                 | 0.023    | SKI       | 0.6                                 | 0.018    |
| FAS       | 1.6                                 | 0.019    | SMAD3     | 0.6                                 | 0.016    |
| FN1       | 0.3                                 | 0.028    | SMAD7     | 0.4                                 | 0.031    |
| ICAM1     | 13.9                                | 0.002    | TFRC      | 0.6                                 | 0.04     |
| ICAM1     | 22.6                                | 0.002    | TGFB1     | 0.6                                 | 0.009    |
| IKBKB     | 0.8                                 | 0.037    | TNFRSF18  | 1.6                                 | 0.007    |
| IL12A     | 6.3                                 | 0.005    | TNFRSF1A  | 0.7                                 | 0.014    |
| IL1A      | 11.1                                | 0.02     | TNFRSF1B  | 2.1                                 | 0.045    |

* Paired T-test by StatMiner.
Supplemental Material, Table 6. Ingenuity toxicity list of functions and pathways relevant to asbestos exposure, based on the differential expression of 32 genes in SAE cells following 48 h asbestos treatment.

| Ingenuity Toxicity Lists                        | Ratio | P Value     | Molecules Involved                                                                                                                                 |
|------------------------------------------------|-------|-------------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| Hepatic Fibrosis                               | 0.11  | 0.0000015   | VEGFA, IL8, IL1A, ICAM1, FN1, CCL2, CSF1, IL6, TNF                                                                                                 |
| LXR/RXR Activation                             | 0.09  | 0.00021     | IL1A, CCL2, NFKB2, IL6, TNFRSF1B, TNF                                                                                                             |
| Hepatic Stellate Cell Activation               | 0.14  | 0.00027     | IL8, CCL2, NFKB2, IL6, TNF                                                                                                                       |
| Hepatic Cholestasis                            | 0.04  | 0.0012      | IL8, IL1A, NFKB2, IL6, TNFRSF1B, TNF                                                                                                             |
| Pro-Apoptosis                                  | 0.10  | 0.0027      | BAX, TNFRSF1B, TNF, FAS                                                                                                                        |
| NFκB Signaling Pathway                         | 0.04  | 0.0036      | IL1A, LTA, NFKB2, TNFRSF1B, TNF                                                                                                                   |
| Oxidative Stress                               | 0.07  | 0.0047      | ICAM1, NFKB2, IL6, TNF                                                                                                                         |
| Aryl Hydrocarbon Receptor Signaling            | 0.03  | 0.0067      | IL1A, BAX, NFKB2, IL6, TNF                                                                                                                       |
| Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα | 0.04  | 0.011       | IL1A, NFKB2, TNFRSF1B, TNF                                                                                                                       |
| PPARα/RXR Activation                           | 0.02  | 0.029       | PLCD1, PLCE1, NFKB2, IL6                                                                                                                         |
| PXR/RXR Activation                             | 0.03  | 0.12        | IL6, TNF                                                                                                                                          |
| FXR/RXR Activation                             | 0.02  | 0.14        | IL1A, TNF                                                                                                                                          |
| p53 Signaling                                  | 0.02  | 0.15        | BAX, BCL2                                                                                                                                         |
| RAR Activation                                 | 0.02  | 0.21        | VEGFA, NFKB2                                                                                                                                       |
| LPS/IL-1 Mediated Inhibition of RXR Function    | 0.01  | 0.28        | TNFRSF1B, TNF                                                                                                                                       |
| Positive Acute Phase Response Proteins         | 0.03  | 0.29        | HMOXI                                                                                                                                             |
| Anti-Apoptosis                                 | 0.03  | 0.29        | BCL2                                                                                                                                               |
| Hypoxia-Inducible Factor Signaling             | 0.01  | 0.41        | VEGFA                                                                                                                                             |
| TR/RXR Activation                              | 0.01  | 0.44        | ADRB1                                                                                                                                             |
| Oxidative Stress Response Mediated by Nrf2     | 0.005 | 0.62        | HMOXI                                                                                                                                             |

**Note:** IPA-Tox analysis allows assessing the toxicity of asbestos by generating a list of tox functions and/or pathways relevant to asbestos exposure. **Ratio** = the number of “genes of interest” in the current study that belong to a given pathway, divided by the total number of genes that make up the pathway (obtained from the existing literature); for example, a ratio of 0.025 indicates that 2.5% of the total gene molecules in a given pathway were also found in this study. **P-value:** the p-value tells the significance of the association between a specific pathway and the genes of interest in this study.
**Supplemental Material, Figure 1.** Characterization of parental and \( \rho^0 \) SAE cells. 

**A.** The morphology of parental and \( \rho^0 \) SAE cells under optic microscope. *Bar*, 50\( \mu \)m. *Right*, enlarged image of boxed area (*left*). 

**B.** Growth curves of parental and \( \rho^0 \) SAE cells. The population doubling time of each cell line was consistent for cells at different passages. 

**C.** Mitochondrial morphology. The mitochondria are fragmented in \( \rho^0 \) cells but elongated and filamentous in parental cells. The green fluorescence indicates mitochondria stained with an antibody against pyruvate dehydrogenase (PDH), a mitochondrial enzyme. *Bar*, 10 \( \mu \)m. Images were captured with fluorescence microscopy (Olympus Bh-2 equipped with Olympus MicroSuite FIVE software). 

**D.** Toxicity of chrysotile and crocidolite asbestos fibers on \( \rho^0 \) and parental SAE cells. The cellular toxicity of asbestos was indicated by survival fraction (percentage of survival over untreated controls) measure with the CyQuant cell proliferation Assay kit. Asbestos and glass fibers exposure duration: 72 hours. Data (mean ± SD) from average of three independent experiments.
Supplemental Material, Figure 2. Network of genes mediated by asbestos after 48 hours of treatment in ρ0 SAE cells. A highly interconnected network of 12 asbestos-responsive genes (P<0.05) was constructed by IPA, based on direct interactions stored in the Ingenuity Knowledge Base, which is a collection of experimentally confirmed relationships between molecules. Color scale bar indicates the fold change in gene expression level, green: down regulation, red: up regulation. Un-colored molecules: expression levels were not examined in the current study. Note: “Ho” is a group/complex suggested by IPA to contain gene(s) examined by our study. “TNF” and “HRH2” were not significantly down- and up- regulated by asbestos according to StatMiner (P value = 0.03 and 0.26, respectively; but suggested by IPA to be affected by asbestos treatment based on the input of 12 interconnected asbestos-responsive genes (this Figure and Supplemental Material, Table 4).
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