Genomic analysis of human lung fibroblasts exposed to vanadium pentoxide to identify candidate genes for occupational bronchitis

Jennifer L Ingram, Aurita Antao-Menezes, Elizabeth A Turpin, Duncan G Wallace, James B Mangum, Linda J Pluta, Russell S Thomas and James C Bonner*

Address: The Hamner Institutes for Health Sciences, Research Triangle Park, North Carolina 27709, USA

Email: Jennifer L Ingram - jennifer.ingram@duke.edu; Aurita Antao-Menezes - amenezes@ciit.org; Elizabeth A Turpin - eturpin@embrex.com; Duncan G Wallace - wallace@ciit.org; James B Mangum - james.b.mangum@gsk.com; Linda J Pluta - lpluta@ciit.org; Russell S Thomas - rthomas@ciit.org; James C Bonner* - jbonner@ciit.org

* Corresponding author

Abstract

Background: Exposure to vanadium pentoxide (V$_2$O$_5$) is a cause of occupational bronchitis. We evaluated gene expression profiles in cultured human lung fibroblasts exposed to V$_2$O$_5$ in vitro in order to identify candidate genes that could play a role in inflammation, fibrosis, and repair during the pathogenesis of V$_2$O$_5$-induced bronchitis.

Methods: Normal human lung fibroblasts were exposed to V$_2$O$_5$ in a time course experiment. Gene expression was measured at various time points over a 24 hr period using the Affymetrix Human Genome U133A 2.0 Array. Selected genes that were significantly changed in the microarray experiment were validated by RT-PCR.

Results: V$_2$O$_5$ altered more than 1,400 genes, of which ~300 were induced while >1,100 genes were suppressed. Gene ontology categories (GO) categories unique to induced genes included inflammatory response and immune response, while GO categories unique to suppressed genes included ubiquitin cycle and cell cycle. A dozen genes were validated by RT-PCR, including growth factors (HBEGF, VEGF, CTGF), chemokines (IL8, CXCL9, CXCL10), oxidative stress response genes (SOD2, PIPOX, OXR1), and DNA-binding proteins (GAS1, STAT1).

Conclusion: Our study identified a variety of genes that could play pivotal roles in inflammation, fibrosis and repair during V$_2$O$_5$-induced bronchitis. The induction of genes that mediate inflammation and immune responses, as well as suppression of genes involved in growth arrest appear to be important to the lung fibrotic reaction to V$_2$O$_5$.

Background

Occupational exposure to vanadium pentoxide (V$_2$O$_5$) has been associated with an increased incidence of chronic obstructive airway disease and a reduction in lung function [1]. V$_2$O$_5$ is the most common commercial form of vanadium and is the primary form found in industrial exposure situations [2]. Occupational exposure to V$_2$O$_5$ occurs during the cleaning of oil-fired boilers and fur-
naces, during handling of catalysts in chemical plants, and during the refining, processing, and burning of vanadium-rich fossil fuels [3].

We previously reported that V₂O₅ causes airway disease in rats that is similar to the pathology of asthma and bronchitis in humans [4]. These pathologic changes include mucus cell hyperplasia, increased airway smooth muscle mass, and peribronchiolar fibrosis. Lung fibroblasts are thought to play a major role in V₂O₅-induced airway remodeling in vivo, as these cells proliferate around airways following injury and deposit collagen which defines the airway fibrotic lesion [4,5].

Vanadium compounds exert cellular stress via inhibition of protein tyrosine phosphatases (PTPs) in cells [6] and through the generation of reactive oxygen species [7,8]. In particular, vanadium compounds have been shown to stimulate release of H₂O₂ in several pulmonary cell types, including alveolar macrophages [9], human lung epithelial cells [10], and human lung fibroblasts [11]. Vanadium-induced oxidative stress has been reported to increase the phosphorylation of MAP kinases through the epidermal growth factor receptor (EGFR) [12] and stimulate activation of multiple transcription factors including p53 [13], AP-1 [14], NF-κB [15] and STAT-1 [8]. These transcription factors play major roles in cell proliferation, apoptosis, differentiation, and the induction of pro-inflammatory mediators. These cellular responses, in turn, determine the overall pathologic outcomes (e.g., inflammation, fibrosis) that lead to the development of V₂O₅-induced bronchitis.

While much is known about signal transduction pathways that are activated by vanadium-induced oxidative stress, much less is known about genes that are regulated by these signaling pathways. In this study, we investigated V₂O₅-induced gene expression in cultured normal human lung fibroblasts using microarray analysis in order to gain a better understanding of the genes that mediate the pathogenesis of fibrosis.

**Methods**

**Cell culture and materials**

Normal adult human lung fibroblasts (ATCC 16 Lu) were purchased from American Type Culture Collection (Rockville, MD). Fibroblasts were seeded into 175 cm² plastic culture flasks and grown to confluence in 10% fetal bovine serum (FBS)/Dulbecco’s modified Eagle’s medium (DMEM), then trypsin-activated, and seeded into 150 mm dishes. Confluent monolayers were rendered quiescent for 24 hrs in serum-free defined medium (SFDM) that consisted of Ham’s F-12 medium with 0.25% BSA with an insulin/transferrin/selenium supplement. Cells were treated with 10 μg/cm² vanadium pentoxide, V₂O₅ (Aldrich Chemical, Milwaukee, WI) or SFDM and RNA was harvested from the fibroblast cultures at 1, 4, 8, 12 and 24 hrs post-treatment. We previously reported that this dose of V₂O₅ causes minimal cytotoxicity (<10% by lactate dehydrogenase assay) and yet induces H₂O₂ production, activates intracellular signaling pathways (e.g., MAP kinases), and upregulates growth factor production by human lung fibroblasts [11]. RNA from an SFDM control was harvested at each of these time points to normalize the V₂O₅ treatment at the same corresponding time point. Three replicate arrays were analyzed for SFDM and V₂O₅ treatment groups at each of the five time points tested.

**Microarray hybridizations and data analysis**

Human lung fibroblast RNA was isolated using RNeasy columns (Qiagen, Valencia, CA). RNA quality was verified by spectrophotometry and gel electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Probe preparation and hybridization to the microarray was performed in the CIIT Gene Expression Core Facility using standard Affymetrix procedures. Double-stranded cDNA was synthesized from RNA using an oligo-dT24-T7. Biotinylated cRNA was synthesized from an aliquot of the cDNA template using the T7 RNA Transcript Labeling Kit (ENZO Diagnostics, Farmingdale NY). The labeled cRNA was then fragmented, hybridized to Affymetrix Human Genome U133A 2.0 arrays (Affymetrix, Santa Clara, CA), and stained using phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR). Gene expression results have been deposited in the National Center for Biotechnology Information (NCBI) Expression Omnibus database [16](Accession Number GSE5339).

**Statistical analysis and data processing**

The microarray data were preprocessed using RMA with a log base 2 (log2) transformation [17]. Statistical analysis of the data was performed in R using the affylinGUI package [18,19]. To identify genes with significant changes in expression following V₂O₅ exposure, all treatment groups were analyzed using a linear model with contrasts between untreated fibroblasts and V₂O₅-exposed fibroblasts at each time point. Genes from all of the five gene lists were combined for the final analysis. Probability values were adjusted for multiple comparisons using a false discovery rate of 5% (FDR ≤ 0.05) [20]. Genes identified as statistically significant were subject to an additional filter by selecting only those genes that exhibited a ≥ 2-fold change from the untreated fibroblasts. Analysis of gene ontology (GO) categories was performed using NIH DAVID [21]. Statistical significance of the GO results was assessed using a hypergeometric test [21]. GO category hierarchy was obtained using AmiGO [22] and used to discard general categories from the DAVID analysis within...
the first three levels. Data for genes changed more than 2-fold were clustered using Cluster 3.0 [22] and visualized using the Maptree Software program [24].

**Real Time quantitative RT-PCR**

Total RNA from human lung fibroblasts was isolated using the Qiagen RNeasy Miniprep kit (Valencia, CA). One or two micrograms of total RNA was reverse transcribed at 48°C for 30 minutes using Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) in 1 × RT buffer, 5.5 mM MgCl₂, 0.5 µM of each dNTP, 2.5 µM of random hexamers, and 0.4 U/µL RNase inhibitor in a volume of 100 µl. One hundred nanograms of the RT product was amplified using Taqman Gene Expression Assays on the Applied Biosystems 7700 Prism® Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR conditions and data analysis were performed according to the manufacturer’s protocol described in User bulletin no.2, Applied Biosystems Prism 7700 Sequence Detection System. All samples were run in triplicate. Gene expression was measured by the quantitation of cDNA converted from mRNA corresponding to VEGF, CTGF, HBEGF, IL8, CXCL9, CXCL10, PIPOX, OXR1, SOD2, STAT1, GAS1, and EGR1 relative to the untreated control groups and normalized to 18S. 18S expression was not significantly changed in the microarray experiment and therefore served as an appropriate housekeeping gene. Relative quantitation values \(2^{-\Delta\Delta CT}\) were expressed as fold-change.

**Results**

Exposure of human lung fibroblasts to V₂O₅ resulted in significantly altered expression of over 1400 genes on the Affymetrix Human Genome U133A 2.0 Array. The majority of significantly changed genes were suppressed by V₂O₅ exposure over the 24 hr time course. Four major temporal patterns of gene expression were identified by hierarchical clustering analysis; progressively induced genes (Fig. 1A and 1B), genes that were induced in a biphasic manner (Fig. 1C), progressively suppressed genes (Fig. 1D) and early induced, late suppressed genes (Fig. 1E). Examples of genes from each of these temporal categories are shown in Fig. 2. The cellular localization and functions of selected genes from each of these categories is shown in Table 1.

An analysis of the biological processes (gene ontology categories) affected by V2O5 exposure in human lung fibroblasts was performed using the NIH DAVID program [21]. This analysis revealed that certain GO categories were unique to V2O5-induced genes, including chemotaxis, inflammatory response, immune response, and cell-cell signaling (Table 2). GO categories that were unique to suppressed genes included ubiquitin cycle, cell cycle, DNA repair, nuclear transport, and programmed cell death. A few categories such as RNA processing were common to induced and suppressed genes.

While analysis of GO biological processes was useful in assessing the overall numbers of significantly changed genes in various functional categories, we selectively grouped genes that have been shown to play important roles in various aspects of tissue injury, repair, and remodeling. These categories included A) cytokines and chemokines, B) growth factors, C) STAT signaling, D) cell cycle regulation, E) oxidative stress, and F) TGF-β signaling (Fig. 3). The functions and cellular localization of representative genes from each of these categories is shown in Table 3. A number of cytokines and chemokines were induced over the time course, including IL8, IL-6, CCL8, CXCL9, and CXCL10, while IL15 was suppressed in a time-dependent manner (Fig. 3A). VEGF, HGF, and HBEGF were progressively induced, while FGF2 and FGF9 were suppressed (Fig. 3B). CTGF was induced early (4 hrs) and suppressed late. Members of the STAT signaling pathway were differentially regulated (Fig. 3C). IRF-1 was induced in a biphasic manner. SOCS3 was progressively induced over the time course, while SOCS1 and IFNGR were progressively suppressed. Genes encoding cell cycle regulation were mainly suppressed, including CDKN1B and CDKN1C, which function to inhibit cell cycle progression (Fig. 3D). Oxidative stress genes were differentially regulated. In particular, SOD2 and PIPOX, which function in peroxide generation, were progressively induced (Fig. 3E). OXR1 and OXSR1, which are protective against oxidative stress, were suppressed. Genes involved in TGF-β signaling and collagen deposition were suppressed, including TGFß2, SMAD1, SMURF1, COL1A1, COL1A2, and COL3A1 (Fig. 3F).

Taqman quantitative real time RT-PCR was used to validate a dozen selected genes that were induced or suppressed by V₂O₅ exposure. We chose to validate 3 genes from each of the following categories (growth factors, chemokines, transcription factors, oxidative stress) that appear to have important roles in inflammation, repair, or fibrosis. The results obtained with Taqman quantitative RT-PCR closely mirrored the patterns of temporal induction or suppression observed in the microarray experiment (Fig. 4).

**Discussion**

Occupational exposure to vanadium oxides has been associated with an increased incidence of obstructive airway disease and a reduction in lung function [1]. In the present study, we investigated the temporal expression of genes in normal human lung fibroblasts exposed V₂O₅,
We previously reported that 10 µg/cm² V₂O₅, the same dose used in our microarray experiment, causes minimal cytotoxicity (<10%) to fibroblasts or epithelial cells over a 24 hr time period [10,11]. This concentration of V₂O₅ also causes several well-defined phenotypic changes in lung fibroblasts including a marked increase in H₂O₂ by fibroblasts [11], phosphorylation of the signal transducer and activator of transcription (STAT-1) [8], and increased expression of heparin-binding EGF-like growth factor, HBEGF [11]. Our current study identified genes regulated by V₂O₅ that could play potentially important roles in oxidative stress, inflammation, growth, and apoptosis during V₂O₅-induced lung injury, remodeling and repair. Moreover, our investigation suggests that fibroblasts play an important role in orchestrating the responses of other pulmonary cell types, including neutrophils, airway epithelial cells, lymphocytes, and endothelial cells. The postulated roles of selected genes that were validated by RT-PCR in mediating V₂O₅-induced inflammation, repair, and fibrosis are illustrated in Fig. 5.

A variety of genes encoding cytokines and chemokines were induced or suppressed by V₂O₅. For example, V₂O₅ induced IL8 and IL6, which play important roles in acute inflammation. We validated the strong induction of IL8 mRNA by RT-PCR. Vanadium rich oil fly ash has been
reported to increase IL8 and IL6 mRNA and protein expression in normal human airway epithelial cells [25,26]. Moreover, workers exposed to vanadium-rich fuel oil ash have increased IL8 protein in nasal fluid [27]. Chemokines induced by V2O5 could play important roles in the immune response. Notably, V2O5 induced CXCL9 (Mig) and CXCL10 (inducible protein-10), both of which were validated by RT-PCR. CXCL9 and CXCL10 are STAT1-dependent chemokines that function in the recruitment of lymphocytes [28]. We previously showed that V2O5 activates STAT1 in lung fibroblasts [8] and mice deficient in STAT1 are susceptible to pulmonary fibrosis [29]. Moreover, we have observed intratracheal V2O5 exposure in rats causes lymphocytic accumulation surrounding airways and small blood vessels, as well causing proliferation of lymphocytes within the bronchus-associ-
After V2O5 treatment remain unclear. We have reported differences in the expression of exposed by intratracheal instillation [30]. The temporal due to the delayed induction of a transcriptional regulator localization and functions (See Fig. 2).

The temporal induction of a transcriptional regulator in fibroblasts and then suppressed. We have also reported that the early induction of HBEGF is due to peroxide dependent activation of MAP kinases [11]. We have also observed that V2O5-induced CTGF expression requires MAP kinases (Ingram and Bonner, unpublished observation). The late induction of HBEGF and VEGF could be due to the delayed induction of a transcriptional regulator gene that is increased in response to V2O5-induced oxidative stress. One such transcriptional regulator that serves as a master switch for growth factor induction is the early growth response (EGR1) gene. EGR1 was significantly induced at 4 and 24 hr following V2O5 treatment in both microarray and RTPCR experiments. EGR1 is induced by a variety of factors including cellular stress and functions as a transcriptional regulator to increase the expression of growth factor genes such as VEGF [33].

Table 1: Temporal expression categories of selected genes significantly induced or suppressed by V2O5 exposure and their cellular localization and functions (See Fig. 2).

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| Hs.25590   | STC1        | Stanniocalcin | Secreted    | Cellular Metabolism |
| Hs.448611  | PBEF1       | Pre-B Cell Colony Enhancing Factor 1 | Secreted    | Inflammation |
| Hs.78913   | CXC3R1      | Chemokine (C-X3-C motif) Receptor 1 | Membrane    | Inflammation |
| Hs.515258  | GDF15       | Growth and Differentiation Factor-15 | Secreted    | Growth Inhibition |
| Hs.471221  | KLF7        | Kruppel-like factor 7 | Nuclear | Transcriptional Regulation |
| Hs.525704  | JUN         | V-jun sarcoma virus 17 oncogene | Nuclear | Transcriptional Regulation |

**Progressively Induced Genes**

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| Hs.65029   | GAS1        | Growth Arrest Specific Gene 1 | Nuclear | Growth Arrest and Apoptosis |
| Hs.519162  | BTG2        | B-Cell Translocation Gene 2 | Nuclear | Growth Arrest |
| Hs.255935  | BTG1        | B-Cell Translocation Gene 1 | Nuclear | Growth Arrest |
| Hs.8375    | TRAF4       | TNF Receptor-Associated Factor | Membrane | Inflammation/Immunity |
| Hs.109225  | VCAM1       | Vascular Cell Adhesion Molecule 1 | Membrane | Cell Adhesion |
| Hs.519090  | MARCKS      | Myristoylated Alamine-rich C Kinase Substrate | Cytoplasmic | Cell Signaling |

**Progressively Suppressed Gene**

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| Hs.298654  | DUSP6       | MAP kinase phosphatase 3 | Cytoplasmic | Cell Signaling |
| Hs.532411  | LYST        | Lysosomal Trafficking Regulator Gene | Cytoplasmic | Cell Signaling |
| Hs.514746  | GATA6       | GATA6 Transcription Factor | Nuclear | Transcriptional Regulation |
| Hs.37982   | NEDD9       | Neural expressed Develop. down-regulated 9 | Membrane | Cell Adhesion |
| Hs.502328  | CD44        | CD44 molecule (Indian blood group) | Membrane | Cell Signaling |
| Hs.59332   | SPRED2      | Sprouty-Related EVH Domain-2 | Cytoplasmic | Cell Signaling |

**Early Induced I/Late Suppressed Genes**

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| Hs.326035  | EGR1        | Early Growth Response-1 Gene | Cytoplasmic/Nuclear | Transcriptional Regulation |
| Hs.534313  | EGR3        | Early Growth Response-3 Gene | Cytoplasmic/Nuclear | Transcriptional Regulation |
| Hs.73853   | BMP2        | Bone Morphogenic Protein-I | Secreted | Cell Differentiation |
| Hs.591241  | CCNT2       | Cyclin T2 | Nuclear | Cell Cycle Regulation |
| Hs.62661   | GBP1        | guanylate-binding protein 1, IFN-inducible | Cytoplasmic | Antiviral Activity |
| Hs.419240  | SLCA2A3     | Solute Carrier Family 2 (GLUT3) | Membrane | Metabolism |

**Biphasic Induced Genes**

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| Hs.25590   | STC1        | Stanniocalcin | Secreted    | Cellular Metabolism |
| Hs.448611  | PBEF1       | Pre-B Cell Colony Enhancing Factor 1 | Secreted    | Inflammation |
| Hs.78913   | CXC3R1      | Chemokine (C-X3-C motif) Receptor 1 | Membrane    | Inflammation |
| Hs.515258  | GDF15       | Growth and Differentiation Factor-15 | Secreted    | Growth Inhibition |
| Hs.471221  | KLF7        | Kruppel-like factor 7 | Nuclear | Transcriptional Regulation |
| Hs.525704  | JUN         | V-jun sarcoma virus 17 oncogene | Nuclear | Transcriptional Regulation |

aGene annotations are from NCBI http://www.ncbi.nlm.nih.gov.
pressing genes encoding anti-mitogenic factors (GAS1, BTG2, CDKN1B, and CDKN1C). In particular, our RT-PCR results validated GAS1 suppression in V2O5-exposed fibroblasts. While the increased expression of growth factors (i.e., VEGF, HBEGF, CTGF) by fibroblasts exposed to V2O5 is likely important in promoting fibroblast growth and survival, the reduced expression of GAS1 by V2O5 could be equally important in promoting fibroblast replication and survival. Moreover, V2O5 progressively suppressed GAS1 over the entire time course of the experiment, indicating sustained loss of growth arrest control when growth factors such as VEGF, HBEGF, and CTGF were maximally induced.

We found that V2O5 induced or suppressed a number of genes that are involved in oxidative stress. Vanadium compounds have been reported to activate several transcription factors and induce the release of inflammatory mediators through the generation of H2O2 [13,14,8]. Also, we previously reported that human lung fibroblasts exposed to V2O5 release micromolar amounts of H2O2 in vitro 12 to 18 hrs after V2O5 exposure [11]. Two genes encoding peroxide-generating enzymes, SOD2 and PIPOX, were validated by RT-PCR. SOD2 was progressively increased over the 24 hr time course of V2O5 exposure. SOD2 serves as a major protective anti-oxidant defense enzyme that converts superoxide anion to H2O2.

Table 2: Functional analysis of genes induced or suppressed by V2O5 in human lung fibroblasts.

| GO ID  | GO Category                                  | Genes | %   | P value   |
|-------|----------------------------------------------|-------|-----|-----------|
|       | Induced Genes                                |       |     |           |
| 0009605 | response to external stimulus               | 32    | 8.47| 1.43E-05  |
| 0009393 | chemotaxis                                  | 13    | 3.44| 6.96E-05  |
| 0009611 | response to wounding                         | 25    | 6.61| 1.81E-04  |
| 0042221 | response to chemical stimulus                | 23    | 6.08| 2.51E-04  |
| 0006950 | response to stress                           | 44    | 11.64| 0.003553  |
| 0006928 | cell motility                                | 15    | 3.97| 0.005005  |
| 0006396 | RNA processing                               | 19    | 5.03| 0.005027  |
| 0008380 | RNA splicing                                 | 11    | 2.91| 0.007903  |
| 0006954 | inflammatory response                        | 13    | 3.44| 0.011869  |
| 0008284 | positive regulation of cell proliferation    | 10    | 2.65| 0.013783  |
| 0006955 | immune response                              | 33    | 8.73| 0.018616  |
| 0007267 | cell-cell signaling                          | 23    | 6.08| 0.042107  |
|       | Suppressed Genes                             |       |     |           |
| 0045449 | regulation of transcription                  | 298   | 19.34| 3.61E-25  |
| 0006512 | ubiquitin cycle                              | 81    | 5.26| 1.16E-10  |
| 0006391 | RNA processing                               | 72    | 4.67| 6.25E-10  |
| 0007049 | cell cycle                                  | 113   | 7.33| 4.42E-08  |
| 0006974 | response to DNA damage stimulus              | 52    | 3.37| 1.13E-07  |
| 0006295 | DNA metabolism                               | 94    | 6.10| 2.23E-06  |
| 0006281 | DNA repair                                   | 43    | 2.79| 1.23E-05  |
| 0008380 | RNA splicing                                 | 33    | 2.14| 2.56E-05  |
| 0007243 | protein kinase cascade                       | 50    | 3.24| 3.39E-05  |
| 0051301 | cell division                                | 31    | 2.01| 2.71E-04  |
| 0051169 | nuclear transport                            | 23    | 1.49| 6.27E-04  |
| 0016310 | phosphorylation                              | 88    | 5.71| 8.76E-04  |
| 0019538 | protein metabolism                           | 311   | 20.18| 0.001149  |
| 0030518 | steroid hormone receptor signaling pathway   | 13    | 0.84| 0.001328  |
| 0050658 | RNA transport                                | 12    | 0.78| 0.002917  |
| 0012501 | programmed cell death                        | 76    | 4.93| 0.003907  |
| 0001558 | regulation of cell growth                    | 22    | 1.43| 0.004779  |
| 0016568 | chromatin modification                       | 22    | 1.43| 0.005351  |
| 0007259 | JAK-STAT cascade                             | 9     | 0.58| 0.008321  |
| 0007050 | cell cycle arrest                            | 14    | 0.91| 0.013090  |
| 0016055 | Wnt receptor signaling pathway               | 18    | 1.17| 0.020398  |
| 0015031 | protein transport                            | 65    | 4.22| 0.034144  |
| 0008286 | insulin receptor signaling pathway           | 6     | 0.39| 0.039295  |
| 0007249 | I-kappaB kinase/NF-kappaB cascade             | 18    | 1.17| 0.042224  |

* GO analysis performed using NIH DAVID [http://david.abcc.ncifcrf.gov](http://david.abcc.ncifcrf.gov).
* Gene ontology ID numbers obtained from AmiGO [http://www.genedb.org/amigo/perl/go.cgi](http://www.genedb.org/amigo/perl/go.cgi).
* % of total induced or suppressed genes.
Table 3: Cellular localization and functions of genes regulated by V$_2$O$_5$ grouped by functional categories (See Fig. 3).

| Accession# | Gene Symbol | Gene Name | Localization | Function |
|------------|-------------|-----------|--------------|----------|
| **Cytokines and Chemokines** | | | | |
| Hs.512234  | IL6         | Interleukin-6 (interferon beta2) | Secreted  | Inflammation |
| Hs.624     | IL8         | Interleukin-8     | Secreted  | Neutrophil Chemotaxis |
| Hs.168132  | IL15        | Interleukin-15    | Secreted  | T Lymphocyte Proliferation |
| Hs.271387  | CCL8        | CC Chemokine Ligand 8 | Secreted  | Neutrophil Chemotaxis |
| Hs.77367   | CXCL9       | Chemokine (C-X-C motif) Ligand 9 (Mig) | Secreted  | Inflammation |
| Hs.632586  | CXCL10      | Chemokine (C-X-C motif) Ligand 10 (IP-10) | Secreted  | Inflammation |
| **Growth Factors** | | | | |
| Hs.73793   | VEGF        | Vascular Endothelial Cell Growth Factor | Secreted  | Endothelial Cell Growth |
| Hs.396530  | HGF         | Hepatocyte Growth Factor | Secreted  | Epithelial Cell Growth |
| Hs.799     | HBEGF       | Heparin-Binding EGF-like Growth Factor | Membrane/Secreted | Fibroblast Growth |
| Hs.591346  | CTGF        | Connective Tissue Growth Factor | Secreted  | Collagen Synthesis |
| Hs.111     | FGF9        | Fibroblast Growth Factor-9 | Membrane/Secreted | Fibroblast Growth |
| Hs.284244  | FGF2        | Fibroblast Growth Factor-2 | Membrane/Secreted | Fibroblast Growth |
| **STAT Signaling** | | | | |
| Hs.591081  | JAK2        | Janus Activated Kinase-2 | Membrane  | STAT Phosphorylation |
| Hs.436061  | IRF1        | Interferon-Regulatory Factor-1 | Cytoplasmic/Nuclear | Transcriptional Regulation |
| Hs.527973  | SOCS3       | Suppressor of Cytokine Signaling-3 | Cytoplasmic | Cell Signaling |
| Hs.50640   | SOCS1       | Suppressor of Cytokine Signaling-1 | Cytoplasmic | Cell Signaling |
| Hs.470943  | STAT1       | Signal Transducer Activator of Transcription | Cytoplasmic | Growth Arrest and Apoptosis |
| Hs.520414  | IFNGR1      | Interferon Gamma Receptor-1 | Membrane  | Cell Signaling |
| **Cell Cycle Regulation** | | | | |
| Hs.238990  | CDKNI B     | Cyclin-Dependent Kinase Inhibitor-1B (Kip1) | Nuclear  | Cell Cycle Arrest |
| Hs.106070  | CDKN1C      | Cyclin-Dependent Kinase Inhibitor-1C (Kip2) | Nuclear  | Cell Cycle Arrest |
| Hs.525324  | CDKN2C      | Cyclin-Dependent Kinase Inhibitor-2C | Nuclear  | Cell Cycle Arrest |
| Hs.557646  | CDK9        | Cyclin-Dependent Kinase-9 | Nuclear  | Transcriptional Regulation |
| Hs.184298  | CDK7        | Cyclin-Dependent Kinase-7 | Nuclear  | Transcriptional Regulation |
| Hs.13291   | CCNG2       | Cyclin G2 | Nuclear  | Cell Cycle Arrest |
| **Oxidative Stress** | | | | |
| Hs.475970  | OXR1        | Oxidative Stress Response 1 | Cytoplasmic | Intracellular Kinase |
| Hs.487046  | SOD2        | Superoxide Dismutase 2 (SOD2) | Cytoplasmic | Peroxide Generation |
| Hs.148778  | OXR1        | Oxidative Resistance 1 | Cytoplasmic | Anti-Oxidant |
| Hs.462585  | PIPOX       | Pimelic Acid Oxidase | Cytoplasmic | Peroxide Generation |
| Hs.465870  | KEAP1       | Kelch-like ECH-associated protein 1 | Cytoplasmic | Redox Homeostasis |
| Hs.406515  | NQO1        | NAD(P)H:quinone oxidoreductase 1 | Cytoplasmic | Redox Homeostasis |
| **TGF-beta Signaling and Collagen** | | | | |
| Hs.133379  | TGF2        | Transforming Growth Factor beta-2 | Secreted  | Matrix Synthesis, Immunity |
| Hs.519005  | SMAD1       | mothers against DPP homolog 1 | Cytoplasmic | Cell Signaling |
| Hs.189329  | SMURF1      | Smad Ubiquitin Regulatory Factor-1 | Cytoplasmic | Cell Signaling |
| Hs.489142  | COL1A2      | Collagen 1A2 | Secreted  | Structural Protein |
| Hs.172928  | COL1A1      | Collagen 1A1 | Secreted  | Structural Protein |
| Hs.443625  | COL3A1      | Collagen 3A1 | Secreted  | Structural Protein |

*Gene annotations are from NCBI [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).*
Gene expression profiles of selected genes for six functional categories. Fold changes in gene expression over the time course of the experiment are shown on a log₂ scale. A) Cytokines and Chemokines, B) Growth Factors, C) STAT Signaling, D) Cell Cycle Regulation, E) Oxidative Stress, and F) TGF-β Signaling. The cellular localization and function of each of these genes are shown in Table 3.
Validation of selected genes by Taqman quantitative RT-PCR. RNA was isolated from human lung fibroblasts treated with 10 µg/cm² V2O5 at the indicated time points and RT-PCR performed as described in Methods. Three genes from four categories were validated: growth factors (top row: VEGF, HBEGF, CTGF), chemokines (second row: IL8, CXCL9, CXCL10), transcription factors (third row: Egr1, STAT1, GAS1), and oxidative stress genes (bottom row: PIPOX, OXR1, SOD2). The data for each gene was normalized against 18S housekeeping gene and expressed as the mean ratio. Data are representative of at least two replicate experiments and expressed as the mean ± sem of triplicate dishes of cells. The temporal pattern of each V2O5-altered gene validated by RT-PCR is compared with the result obtained from the microarray experiment (open diamonds).
[39]. V₂O₅ undergoes redox chemistry to generate superoxide anion, so it is possible that SOD2 plays a role in reducing V₂O₅-induced lung injury. L-pipeolate oxidase (PIPOX), a peroxisomal oxidase, was also progressively induced by V₂O₅. PIPOX utilizes molecular oxygen as a substrate with H₂O₂ as a product [40]. While V₂O₅ induces genes that generate peroxide (SOD2, PIPOX), we also validated suppression of the oxidative resistance gene (OXR1). Volkert and colleagues discovered the human OXR1 gene using a functional genomics approach in a search for genes that function in protection against oxidative damage [41]. While OXR1 is protective against oxidative stress, the precise function of this gene is not well understood. Because OXR1 is protective against oxidative injury, suppression of this gene could contribute to V₂O₅-induced oxidative stress. Also, the temporal suppression of OXR1 occurs as PIPOX (a pro-oxidative stress gene) is temporally induced.

V₂O₅ causes airway fibrosis in rats in vivo, and it is well known that increased collagen production defines the fibrotic lesion [4]. TGF-β is an essential mediator of collagen production by fibroblasts. Our results showed that TGFB2, along with its associated signaling intermediates SMAD1 and SMURF1, were all progressively suppressed by V₂O₅. Moreover, several major collagen genes (COL1A2, COL1A1, COL3A1) were suppressed as well. These data indicate that V₂O₅ does not directly stimulate fibroblasts to deposit collagen. Instead, it is likely that TGF-β or other factors signals produced by neighboring pulmonary cell types to increase collagen production. TGF-β mRNA is increased in the lungs of rats treated with V₂O₅. Therefore, during V₂O₅-induced fibrogenesis fibroblasts do not appear to be effectors of their own collagen deposition, but likely require other cell types (e.g., macrophages) as a source of TGF-β.

While we used lung fibroblasts in our study, it is highly relevant to consider the effect of V₂O₅ exposure on gene expression by other lung cell types, including epithelial cells. Li and colleagues used microarray analysis to investigate gene expression changes in human bronchial epithelial cells exposed to vanadium or zinc and identified a small set of genes that could be used as biomarkers for discriminating vanadium from zinc [42]. They also reported that IL8 and PTGS2 (COX-2) were induced several-fold by vanadium but not by zinc. IL8 and PTGS2 were also strongly induced in human lung fibroblasts by vanadium.
in our study. In fact, we previously reported that COX-2 null mice are susceptible to V$_2$O$_5$-induced lung fibrosis, which emphasized an important protective role for the PTGS2 gene during fibrogenesis [43].

**Conclusion**

A variety of genes were induced or suppressed in normal human lung fibroblasts by vanadium pentoxide (V$_2$O$_5$) that appear to have important functions in inflammation, fibrosis and repair. Our data suggest that both the induction of genes that mediate cell proliferation and chemotaxis (VEGF, CTGF, HBEGF), as well as suppression of genes involved in growth arrest and apoptosis (GAS1), is important to the lung fibrotic reaction to V$_2$O$_5$. The induction of interferon-inducible, STAT1-dependent chemokines (CXCL9 and CXCL10) could contribute to both suppression of fibroblast proliferation and lymphocyte accumulation. The strong induction of IL8 likely contributes to neutrophilic inflammation. An increase in peroxide-generating enzymes (PIPOX, SOD2) is consistent with H$_2$O$_2$ production by V$_2$O$_5$, while the reduced expression of protective oxidative response genes (e.g., OXR1) could further contribute to oxidative damage. Overall, our study reveals a wide variety of candidate genes that could mediate V$_2$O$_5$-induced airway remodeling after occupational and environmental exposures.

**Abbreviations**

V$_2$O$_5$, vanadium pentoxide; STAT-1, signal transducer and activator of transcription; GAS1, growth arrest specific gene; VEGF, vascular endothelial cell growth factor; CTGF, connective tissue growth factor; CXCL10, Chemokine (C-X-C motif) ligand 10; HB-EGF, heparin-binding epidermal growth factor-like growth factor; PTGS-2, prostaglandin synthase 2; OXR1, oxidative resistance gene; SOD2, superoxide dismutase-2; PIPOX, L-pipocelolate oxidase.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors’ contributions**

JLI and ICB designed the experiments, performed the data analysis, and drafted the manuscript. JLI, AAM, EAT, JBM, and DGW performed cell culture, RNA isolation, and validated changes in selected genes by Taqman quantitative real-time RT-PCR. JLP performed with microarray hybridizations. RST performed statistical analysis on the microarray data. All authors read and approved the final manuscript.

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