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Endothelial effects of emission source particles: Acute toxic response gene expression profiles

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Abstract

Air pollution epidemiology has established a strong association between exposure to ambient particulate matter (PM) and cardiovascular outcomes. Experimental studies in both humans and laboratory animals support varied biological mechanisms including endothelial dysfunction as potentially a central step to the elicitation of cardiovascular events. We therefore hypothesized that relevant early molecular alterations on endothelial cells should be assessable in vitro upon acute exposure to PM components previously shown to be involved in health outcomes. Using a model emission PM, residual oil fly ash and one of its predominant constituents (vanadium-V), we focused on the development of gene expression profiles to fingerprint that particle and its constituents to explore potential biomarkers for PM-induced endothelial dysfunction. Here we present differential gene expression and transcription factor activation profiles in human vascular endothelial cells exposed to a non-cytotoxic dose of fly ash or V following semi-global gene expression profiling of ~8000 genes. Both fly ash and it's prime constituent, V, induced alterations in genes involved in passive and active transport of solutes across the membrane; voltage-dependent ion pumps; induction of extracellular matrix proteins and adhesion molecules; and activation of numerous kinases involved in signal transduction pathways. These preliminary data suggest that cardiovascular effects associated with exposure to PM may be mediated by perturbations in endothelial cell permeability, membrane integrity; and ultimately endothelial dysfunction.

Keywords

Endothelial; Particulate matter; Vascular effects

1. Introduction

Ambient particulate matter (PM) is a heterogeneous mixture of liquid and/or solid materials suspended in the air, whose chemical composition and size vary over time and space. Although the composition of PM varies according to the geographical location of collection, PM contains many common constituent groups such as sulfates, nitrates, elemental carbon, organic carbon,

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pollen, endo-toxins and various metals. From a regulatory perspective, PM in the ambient air is classified into two primary size modes (aerodynamic mass median diameters) based on their aerodynamic properties exploited during collection method. These size modes are known as fine ($PM_{2.5}$) and coarse (PM_{10}–$PM_{2.5}$) mode PM and are generally thought to relate to the primary lung region in which they deposit – fine in the deep lung and coarse in the conducting airways. A third mode of PM has been garnering attention, the so-called ultrafine PM, which are optically <100 nm in diameter. These PM are not directly regulated but they do contribute to fine PM via their agglomeration with and into fine PM.

The bulk of the early epidemiology targeted the respiratory tract as the primary effect organ that yielded measurable health outcomes (both morbidity and mortality). In general, these effects have been found to relate best to ambient fine PM (Pope et al., 2002; Pope and Dockery, 2006). As the health assessments of PM effects evolved, and studies have attempted to refine causality, cardiac-mediated mortality and hospital admissions for cardiovascular diseases or events have risen as prominent outcomes (Brook et al., 2004; Brook, 2005; Kunzli et al., 2005). These cardiovascular outcomes have been linked to direct cardiac and vascular mediated modes of action (e.g., vascular constriction, thrombogenesis). Measurements of cardiac and vascular function in humans exposed to PM and analogous experimental work in animals provide coherent evidence of the potential impacts of PM or its constituents on the heart (Godleski, 2004; Rundell et al., 2007; Cascio et al., 2007).

Various hypotheses have been proposed explaining the biochemical or molecular basis for the cardiovascular effects associated with PM and/or its constituents (Bai et al., 2007). The inflammatory reactions initiated in the lung by particles may trigger release of mediators that can influence the heart directly or associated vasculature. Systemic oxidative stress/ inflammation with effects on cardiovascular tissues might then parallel inflammation in the lungs post exposure (Godleski, 2004; Frampton, 2006). Others propose direct effects of PM or ultrafines due to their translocation from the lungs into the systemic circulation (Oberdorster and Utell, 2002; Oberdorster et al., 2004) and direct transmission to cardiac and cardiovascular tissues. Analogously, constituents of PM that are solubilized in the lung may also enter the circulation (Knuckles and Dreher, 2007; Wallenborn et al., 2007) and directly impact the heart or vasculature. With systemic penetration of constituents and/or vascular inflammation and oxidative stress, endothelial function can be dysregulated with subsequent vasoconstriction or hyperreactivity to normal stimuli (Brook et al., 2002; Knaapen et al., 2001; Nurkiewicz et al, 2004). Vasocon-striction of small pulmonary arteries in rats exposed to concentrated ambient particles (Batalha et al., 1998) and impaired arterial endothelial relaxation with exposure to diesel exhaust particles have been postulated to be mediated by oxidative stress/ inflammation at the endothelial level (Ikeda et al., 1995; Hirano et al., 2003). Oxidative events such as these are potential stimuli to many genes which upon dysregulation can alter function and lead to the panoply of events noted.

To understand the potential direct effects of soluble constituents of PM on endothelial integrity we sought to analyze gene expression profiles in primary human umbilical vein endothelial cells (HUVEC) as a model to generate hypotheses on potential molecular mechanism(s) involved in endothelial dysfunction. The findings may predict potential pathways or candidate genes to develop biomarkers of endothelial alterations in air pollutant-induced cardiovascular effects. Towards this goal, we chose to use a well-characterized model emission source PM, a residual oil fly ash (ROFA) that is rich in transition metals and sulfates (Dreher et al., 1997; Costa and Dreher, 1997; Gavett et al., 1997). This ROFA has been shown to elicit cardiac effects in vivo and in vitro (Costa and Dreher, 1997; Watkinson et al., 1998; Proctor et al., 2006). The transition metals in this combustion PM are known for systemic penetration (Knuckles and Dreher, 2007). Occupational exposure to vanadium-rich environmental pollutants has been reported in power plant workers and boilermakers (Levy et al., 1984;

Woodin et al., 1999, 2000) and has been shown to be a significant constituent of ambient PM in the Northeast (Lippmann et al, 2005). Vanadium along with other transition metals such as Ni, Zn and Fe has been found in ambient PM samples from a number of sources (Dominici et al, 2007). The toxic effects of V in animal models range from cancer to cardiovascular effects (Coderre and Srivastava, 2004; Campen et al., 2001; Huang et al., 2002; Li et al., 2005). To ascertain the potential contribution of V in ROFA in inducing vascular effects and how these effects may be affected by other constituents in ROFA, we hypothesized that ROFA- and V-induced transcriptional alterations would show largely similar immediate effects on endothelial function. We found similarity and dissimilarities between ROFA and V in the expression of genes involved in the endothelial permeability, solute transport and membrane structure consistent with their potential to initiate PM-like alterations in vascular homeostasis and cardiovascular toxicity suggesting V-independent and interactions with other metal constituents.

2. Methods

2.1. Cells

Primary human umbilical vein endothelial cells (HUVEC) were obtained from VEC Technologies Inc., (New York, NY) and grown in complete MCDB-131 culture medium containing 10% fetal bovine serum and supplemented with growth factors and antibiotics. In all the experiments reported here cells from passages six to eight were utilized.

2.2. PM samples

Combustion source residual oil fly ash (ROFA), with a mass median aerodynamic diameter of 1.95 μm and a geometric standard deviation (F_g) of 2.14 collected by Southern Research Institute (Birmingham, AL) downstream of a power plant burning low sulfur number 6 residual oil obtained from Florida power plants and $VS0_4$, $7H_2O$ (K. K. Laboratories, Plainview, NY) were used in these studies.

2.3. Particle exposure

HUVEC were acclimatized for 12 h in serum free MCB-131 medium, washed with room temperature PBS and exposed in a fresh serum free MCDB-131 medium. ROFA particles were suspended in pyrogen-free bacteriostatic saline and mixed gently for 20 min and centrifuged at 10,000 g for 10 min. The aqueous extracts of ROFA and V were diluted in serum free MCDB-131 medium. The cells were exposed to various concentrations of ROFA (0.5 μg-10 μg/ml) or V (0.1 μM-10 μM) or saline for 1, 3 and 8 h.

2.4. Cytotoxicity assay

To determine the cytotoxicity of ROFA and V on HUVEC, a dose response kinetics study was carried out. HUVEC (0.1×10^6) were plated in triplicate in a 12 well plate and used after 48 h when the cells were ~90% confluent. Cells were acclimatized for 12 h in serum free MCB-131 medium, washed with room temperature PBS and exposed in a fresh serum free MCDB-131 medium containing saline, ROFA or V. The cell culture medium was collected (0.3 ml) at 1, 3 and 8 h post exposure was assayed for lactate dehydrogenase (LDH) activity using KoneLab 30 clinical analyzer (Thermo Clinical Lab Systems, Espoo, Finland). The statistically significant difference in LDH activity was derived following Student's *t* test method.

2.5. Microarray analysis

ROFA (1 μg/ml) and V (1 μM)-induced gene expression profiles were derived by exposing HUVEC for 30 min. Prior to exposure, the cells were acclimatized for 12 h in serum free MCB-131 medium. At the end of exposure the cells were washed once with ice-cold PBS and

RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA). The quality of total RNA was assayed by Bio-Analyzer (Agilent Technologies, Palo Alto, CA). Total RNA (15 μg) was enriched to polyA+ RNA using Atlas Pure Total RNA labeling System (Clontech. Palo Alto, CA) and utilized in cDNA labeling reactions to generate ^{33}P-labeled cDNA probe for Atlas Human 8 K plastic microarray (containing 8330 genes derived from NCBI Unigene database, each gene is represented by 80mer oligonucleotide spotted in duplicate) following manufacturer (Clontech., Palo Alto, CA) recommended protocol with modifications (Nadadur and Kodavanti, 2002). The microarrays were hybridized for 12-14 h at 60 °C in plastic microarray hybridization buffer. The microarray was washed following manufacturer recommended protocol and exposed to phosphor screen for 18-24 h. The screens were scanned using Typhoon phosphorimager (Molecular Dynamics, Piscataway, NJ). The gene spots in the scan image were aligned using AtlasImage 2.7 software (Clontech, Palo Alto, CA), and the spot intensities (gene expression) were normalized to the median for background correction. Spot density values were imported to GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA) and analyzed for changes in gene expression. The gene expression patterns were derived with a cutoff value of a >2-fold change for each exposure condition ($N = 3$). Statistically significant genes were identified by 1-way ANOVA (P values of <0.05) without any correction. The groups of genes with a statistically significant change and at least a >2-fold difference compared to the saline-exposed group were generated using Venn diagram approach.

2.6. Transcription factor (TF) array analysis

Activation profile for 54 TFs was assayed using TranSignal Protein/DNA array (Panomics, Inc., Redwood City, CA). Nuclear proteins were extracted from identically exposed cells [for 30 min to saline, ROFA (1 μg/ml) or V (1 μM)]. Cells were washed once with cold PBS, lysed using NE-PER nuclear and cytoplasmic extraction buffer (Pierce, Rockford, IL) according to kit directions. Nuclear protein was quantified using coomasie Plus Protein Reagent (Pierce, Rockford, IL) adapted for use on the KoneLab 30 clinical analyzer (Thermo Clinical Lab Systems, Espoo, Finland). 10 μg of nuclear protein was incubated with the TranSignal Probe mix (Panomics, Inc., Redwood City, CA) and the TF array was hybridized following manufacturer recommended protocols. The array blots were exposed to X-ray film for 30 s and the blot images were scanned using a personal densitometer (Molecular Dynamics, Piscataway, NJ). The density of the TF spots (in duplicate) was quantitated using ImageQuant software (Molecular Dynamics, Piscataway, NJ).

2.7. Computational analysis of transcription factor binding

The biological significance of the gene expression data is often validated by establishing translation of the message to protein. It is also important to link upstream events and stimuli that are responsible for the induction of expression of specific gene(s). These latter linkages can be accomplished *in silico* by bioinformatics. As the data are available for the TFs activated in these studies, identifying TF binding sites on the gene sequence of the induced genes could be carried out using TRANSFAC database analysis. As a pilot project we selected 30 genes induced by either ROFA or V and pulled the gene sequence from GenBank, NCBI, using gene accession # and utilized it to perform via online TRANSFAC search (http://www.cbrc.jp/research/db/TFSEARCH.html). These searches are based on two simple principles: the TF is an identified protein in the species under investigation (human in this case), and the consensus TF binding sequence is known. The results of these analyses will provide the number of TF binding sites with the sequence on the genes of interest. The number of binding sites can be counted and scored. For this analysis we selected the following TFs:AP1, Brn2, USF-1, Stat4/5, CDP and Ets-1 and queried for their binding sites on the genes found induced by either ROFA or V.

3. Results

3.1. Cytotoxicity analysis

The cytotoxic response of HUVEC upon exposure to ROFA (0.5 μg-10 μg/ml) and V (0.1 μM-10 μM or ~0.27-2.7 μg/ml) was analyzed by measuring the leakage of LDH protein into the cell culture medium. The viability of cells 8 h postexposure to ROFA and V was evaluated using the MTT [3-(4,5-dimethylthiazol-2-lyl)-2,5-diphenyltetrazolium bromide] assay, and alterations in cell count (by 0.5%) were not found. The LDH data (8 h post exposures) presented in Fig. 1, suggest concentration dependent cytotoxicity to HUVEC. The dose of ROFA (1.0 μg/ml) and V(1.0 μM) were found to display minimal cytotoxicity at 8 h postexposure based on LDH release assay and by MTT assay (data not shown). These two exposure regimens were selected to investigate the acute exposure-induced gene expression profiles in HUVEC.

3.2. Gene expression profiles

Gene expression profiles were generated with microarray analysis to better understand the immediate response of endothelial cells on direct exposure to an aqueous extract of ROFA or V. Differential gene expression based on fold change (>2.0) indicated almost equal number of genes altered on exposure to either ROFA or V. Of the altered genes, 645 were found induced and 983 genes were found suppressed due to ROFA or V-exposure respectively (Table 1). The Venn diagram approach in the GeneSpring software was utilized to identify exposure-specific differential expression of genes. This analysis also indicated that 119 of induced and 226 of suppressed genes are common to both ROFA and V suggesting their involvement in initiating similar mechanisms of response (Table 1). To derive biological significance and the role for these altered genes in endothelial function, they were subjected to additional classification following Venn diagram approach. This analysis led to the distribution of altered genes into the following families: transporter proteins, signal transduction events, kinases, voltage-dependent signaling, cell adhesion molecules and cytokines/ growth factors (Table 2) according to exposure. The classification of genes in the signal transduction category with this program is broad and includes mediators, receptors and down stream kinases. The list of genes representing five functional clusters that are unique to ROFA (Table 3) or V exposure (Table 4) and common to both (Table 5) are presented in Tables 3–5.

3.3. Transcription factor (TF) activation profiles

Analysis of TranSignal Protein/DNA array data on activated upstream TFs indicated signals well above the background for 36 TFs suggesting basal level of activation. Differential activation (by >2-fold) was observed in 9 TFs in ROFA exposed and 13 TFs in V exposed HUVEC (Table 6). AP-1, AP-2 and Brn3 are the 3 TFs found predominantly activated multifold in HUVEC on exposure to ROFA or V. Activation of AP-1 and AP-2 were 10-fold higher in V compared to ROFA. Four TFs (c-Myb, HNF-4, MEF-1 and USF-1) were found marginally activated and to similar extent in both ROFA and V exposed HUVEC. On the other hand activation of Stat5 was observed only in ROFA exposed cells, while activation of CDP, Ets-1 and p53 were exclusive to V-exposed cells.

3.4. TRANSFAC database analysis

The data presented in Table 7 shows the distribution profile for the TF binding sites on 30 representative genes found induced by either ROFA or V. Each “+” represents one binding site for the respective TF. This analysis allowed establishing a functional relationship between the activation of TF and its potential contribution (by binding) to the putative binding site on the gene. Based on the scores (number of binding sites) it's possible to establish role for a specific TF in the induction of gene. This analysis provides more useful in the characterization

of upstream events involved in the activation of TF by specific exposures that ultimately lead to signaling cascades that are involved in mediating this response.

4. Discussion

Epidemiological and animal toxicology studies provide coherent evidence that endothelial dysfunction is involved in some way with the cardiovascular effects of ambient PM or its toxic constituents. Studies in animals have demonstrated that both particles (Calderon-Garciduenas et al., 2001; Kreyling et al., 2002; Oberdorster et al., 2002) and their leached constituents (Knuckles and Dreher, 2007; Wallenborn et al., 2007) can escape the lung and move into extra-pulmonary tissues. While at present, the role of neurally-mediated endothelial dysfunction cannot be ruled out, the evidence for systemic penetration and the impact of bioavailable or soluble components of PM on endothelial function seems highly possible. Our data obtained from semi-global analysis of transcriptional alterations in a focused collection of gene families in vascular endothelial cells provide credible hypotheses to explore pathways that may mediate observed PM-induced microvascular alterations (Huang et al., 2002; Nurkiewicz et al., 2004; van Eeden et al., 2005).

The involvement of water soluble constituents of PM has been proposed in the elucidation of systemic toxic effects in extra-pulmonary organs. Dreher et al. (2002) by pulmonary deposition (intra-tracheal instillation) of ~345 μg ROFA demonstrated the presence of a plasma concentration of 3.5 μg/ml 3 h post instillation (as estimated by serum V concentrations). Based on these observations Knuckles and Dreher (2007) carried out in vitro studies on rat cardiomyocytes using 3.5 μg/ml ROFA and observed ROFA-induced differential gene expression. We present here for the first time gene expression alterations observed at much lower concentrations of ROFA (1 μg/ml) and V (1.0 μM or ~0.27 μg/ml). The dose of V (1.0 μM; ~0.27 μg/ml) used in the present studies is equivalent to six times of the amount of V present in 1 μg of ROFA (Dreher et al., 1997) or twice the plasma levels as reported by Knuckles and Dreher (2007) with essentially equivalent biological response (in this case equal numbers of LDH units released into the medium 8 h post exposure). This presented an opportunity to understand whether this similarity in biological response is mediated by alterations in the expression of similar sets of genes and (2) whether there are differences in the gene expression profiles that may shed light on the potential interaction among other constituents in ROFA to interfere with V-induced toxicity responses.

Both ROFA and V-induced endothelial cell gene expression alterations are found to be predominantly distributed in the following specific gene families: solute carrier proteins, ATPase mediated transport proteins, voltage-gated channels, kinases, cell adhesion molecules, and extracellular matrix proteins (Tables 3–5). This pattern of altered gene expression profile indicates perturbations in endothelial cell's permeability barrier function, maintenance of electrochemical gradient and subsequent down stream signaling events. Although there is a common set of genes within the gene families, there is a clear exposure-specific differential expression within the members of these gene families. These differences might be anticipated due to the influence of other metals or bioavailable constituents within ROFA. But one important observation to be noted here is that despite differences in the amount of V and potential influences of other constituents within ROFA, the endothelial effects appear to be mediated by regulation of similar gene families. Additional studies with other predominant constituents of ROFA such as Ni or Fe may provide different profiles. But, based on the current data, it can be presumed that endothelial effects observed here appear to be predominantly driven by V-induced gene expression changes. The evaluation of differential activation profile for various TFs in endothelial cells in response to ROFA or V provided insights to validate relationships between TF activation and gene expression following computational approaches using TRNSFAC. Utilizing these *in silico* approaches allows us to postulate with some

confidence the following plausible mechanisms in the acute toxicity response in human vascular endothelial cells.

4.1. Endothelial permeability

The barrier function of endothelium is dependent on the maintenance of endothelial cell permeability properties. The initial response to altered permeability interferes with the transport of solutes across the endothelium. Two different trans-endothelial pathways participate in the passage of solutes, mediated by cell membrane transport proteins that control the uptake and efflux of crucial compounds such as sugars, amino acids, nucleotides, ions and drugs. Transport proteins are of two types: passive and active transporters. Passive transporters or facilitated transporters allow passage of solutes (ex. glucose, amino acids and urea) across the membrane down their electrochemical gradient. Active transporters on the other hand create ion/solute gradients across the membrane utilizing diverse energy-coupling mechanisms. Plasma membrane adenosine triphosphatase (ATPase) family, that includes Na^+, K^+, H^+, K+ and Ca^{2+}- ATPases constitute ion pumps with catalytic activities that are responsible for the maintenance of cellular homeostasis, osmotic balance and intracellular ion composition.

To date there are ~300 known genes coding for solute carrier (SLC) transport proteins that participate in the maintenance of cellular homeostasis. These genes are classified into 43 gene families and five of them represent transport proteins responsible for the transport of metals across the membranes (Hediger et al., 2004). Of the 43 SLC family of transport proteins, expression of 19 family members were found altered in endothelial cells on exposure to ROFA and V. The observation of altered expression of two metal transport proteins, SLC30 and SLC31 responsible for transport of zinc and copper ions suggest their potential interference by ROFA or V. This observation gains further support from the decreased divalent metal transporter-1 expression observed in lung epithelial cells following ROFA and V exposure (Ghio et al., 2005). The differential expression of genes involved in transport of protons (H^+) in mitochondria in ROFA or V-exposed endothelial cells suggest their potential interference with H+ homeostasis mechanisms in mitochondria. The specific mechanisms of metal effects on mitochondrial function are largely unknown. From the current observations a potential role for SLC30 and SLC31 can be hypothesized.

It has been long recognized that metal ions affect ion channels either by blocking the current or modifying the gating functions (Elinder and Arhem, 2003). Dynamic expression of voltage-dependent gated channel plays a key role in the maintenance of active transport of ions, but their expression is found altered in certain pathophysiological conditions (Marijic et al., 2001). Their alteration due to exposure to PM associated metals in ROFA and to V specifically implies that such exogenous exposures may attack the essential energy-generating organelles of the cell. Recently Xia et al. (2007) reported altered mitochondrial energy production in cells exposed to ultrafine particles. However, the mechanisms that mediate this process largely remain unknown.

4.2. Genes involved in signaling pathways

The gene expression data presented in Tables 3–5 list numerous key candidate genes involved in signal transduction pathways such as kinases, growth factors and cytokines. The majority of these genes are part of down stream signaling events mediated by secondary messengers such as calcium ions. The altered expression of genes modulating calcium ion transport (Ca-ATPases, voltage-dependent calcium channel), that are implicated in endothelial cell permeability alterations (Lum and Malik, 1994;Dudek and Garcia, 2001) have the potential to perturb various signaling pathways (Tiruppathi et al., 2002) and eventually injury to endothelial cells (Yao and Garland, 2005). The exposure-specific differential expression of growth factors and associated kinase suggest potential for initiation of cell injury cascade. For example, altered

expression of PAF, PDGF and fibroblast growth factor-17 and associated kinases in ROFA exposed cells. On the other hand, alterations in the expression of VEGF and associated kinases are specific to V-exposed cells. These exposure-specific differential profiles of growth factors and their down stream signaling pathways suggest diverse response pathways involved in the mediation of endothelial injury.

4.3. Endothelial structure and integrity

An intricately organized network of extracellular proteins constitutes the endothelial basement membrane. A balanced interaction of its components confers barrier function and selective permeability properties for endothelium. In endothelial cells, integrins mediate cell-substrate adhesions through binding to collagen, fibronectin, laminin and vitronectin and form focal adhesions and maintain the integrity of the endothelial barrier function (Qiao et al., 1995; Lum and Malik, 1996). With acute exposure to ROFA or V, endothelial cells exhibited altered expression of cell adhesion molecules and extracellular matrix components (Table 3–5). In this category of genes there are few common to ROFA and V, but there is a clear exposure-specific differential expression in many other genes. This differential expression is more obvious within the adhesion molecules and matrix proteins. Although adhesion molecules (ICAM-2, ICAM-3) and collagens are found predominantly altered in ROFA exposure, integrins are the predominant membrane proteins found altered in V-treated cells. These differences suggest possible alterations in matrix protein interrelationships and their potential involvement in loss of endothelial cell membrane integrity and cell structure.

The observation of a differential expression pattern among members within these gene families indicates potential interaction among other metal constituents with V in mediating endothelial toxicity by ROFA. The gene expression data summarized in Fig. 2, suggests a generalized hypothesis whereby the acute systemic exposure of endothelial cells to metals (such as V and perhaps other PM associated metals) may cause perturbations in endothelial cell permeability and homeostasis. The permeability changes appear to be two-fold: altered transport of solutes across the endothelial membrane and ionic imbalance due to alterations in mitochondrial transport. These together influence the endothelial cell integrity and function. The alterations in various SLC, ion channels and voltage-gated channels reported here for the first time in vascular endothelial cells on exposure to ROFA or V may have the potential to induce endothelial permeability changes similar to inflammation-induced changes (Lum and Malik, 1996). Our present observations, though limited and observed in vitro suggest the importance and need for initiating further research in this area to understand potential role of endothelial dysfunction in cardiovascular effects of air pollution. Further, preliminary analysis of data from our ongoing kinetic studies (data not shown) suggests a cascade of events initiated by these permeability changes. The perturbations in endothelial ion channels, solute carriers and transport proteins gain much more importance in the context of age or pathology associated with compromised function in ion channels (Marijic et al., 2001) and potential exacerbation in such susceptible individuals to air pollution. The ion channel functional alterations may also have long-term consequences in repeated or chronic exposures to air pollutants. In addition to ion channel perturbations, the altered expression in members of SLC family may also contribute to adverse effects. For example, ROFA-induced altered expression of SLC6 members (neurotransmitter transporters) may have potential for PM-induced exacerbations in people as evident from the association of hypertension and polymorphism in SLC6A2 gene in Japanese subpopulations (Ono et al., 2003). Additional studies to elucidate role of SLC6 in PM effects will be more beneficial in understanding role for phenotypes associated with this gene in epidemiological studies.

The semi-global gene expression data presented here suggest that ROFA and its toxic metal constituent, V are capable of inducing endothelial injury even at relatively very low

concentrations and that involves temporal changes in the expression of various genes. The computational approaches utilized in our studies linking gene expression changes with TF activation profiles and their association with gene expression provided more confidence in postulating hypotheses for mediation of endothelial changes following exposure to combustion source PM. The acute effects of ROFA or V on endothelial cells is mediated by altered expression of solute carriers, ion channels and voltage-gated channels and subsequent perturbations in endothelial cell permeability and associated changes in cell homeostasis mechanisms. These initial changes play a major role in mediating trans-endothelial permeability changes and can eventually lead to subsequent cell shrinkage or cell death (Bortner and Cidlowski, 2007). Comprehensive understanding of air pollution-induced endothelial alterations will provide increased knowledge on biological mechanisms involved in extra-pulmonary effects of air pollutants in general and cardiovascular effects in particular. Our ongoing studies of kinetic analysis of global gene expression in endothelial cells exposed to ROFA may shed more light on characterizing some of the mechanisms implicated in these acute exposures studies.

In conclusion, these preliminary analyses of global gene expression in endothelial cells on acute (30 min) exposure to ROFA or V indicate the possible involvement of a variety of pathways in mediating the vascular events associated with PM exposure. Our findings suggest that even small amounts of V (and perhaps other metals) may have potential to initiate effects at the molecular level in vascular endothelium. Moreover, exposures used in these studies were very short (30 min) when compared to more conventional in vitro studies with particulate matter. Clearly, there needs to be more thorough analysis of these events in concert with basic pathophysiology to validate the critical mechanisms underlying endothelial dysfunction in PM-induced cardiovascular effects. But, our current studies indirectly provide possible candidate pathways to be explored to understand the vascular effects of PM constituents that can leach into systemic circulation. These acute response signatures derived from in vitro efforts can be extended to the development of potential biomarkers that can be validated by their association in the etiology of cardiovascular diseases. Such an integrated approach will aid in understanding air pollution effects and perhaps in identifying susceptible populations who might be at greatest risk.

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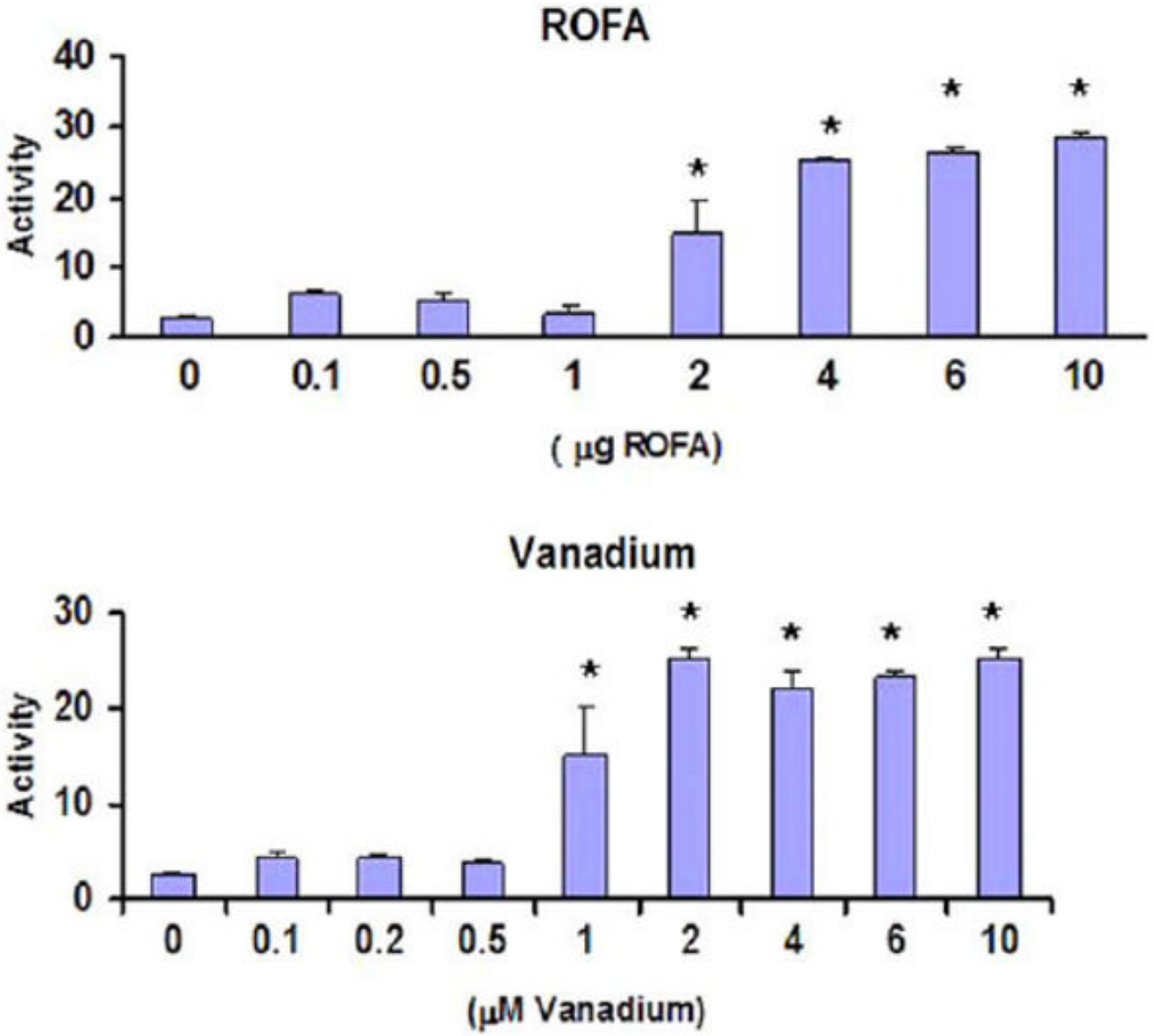

Fig. 1.

The level of lactate dehydrogenase activity was measured in 300 µl aliquots of serum free cell culture medium at 8 h post exposure to various concentrations of ROFA (A) and V (B). The enzyme activity is presented here as units/liter as calculated using reference standard in Kone lab. Each bar represents the mean + -SEM for triplicate samples. Asterisk indicates significantly different from control ($p < 0.05$).

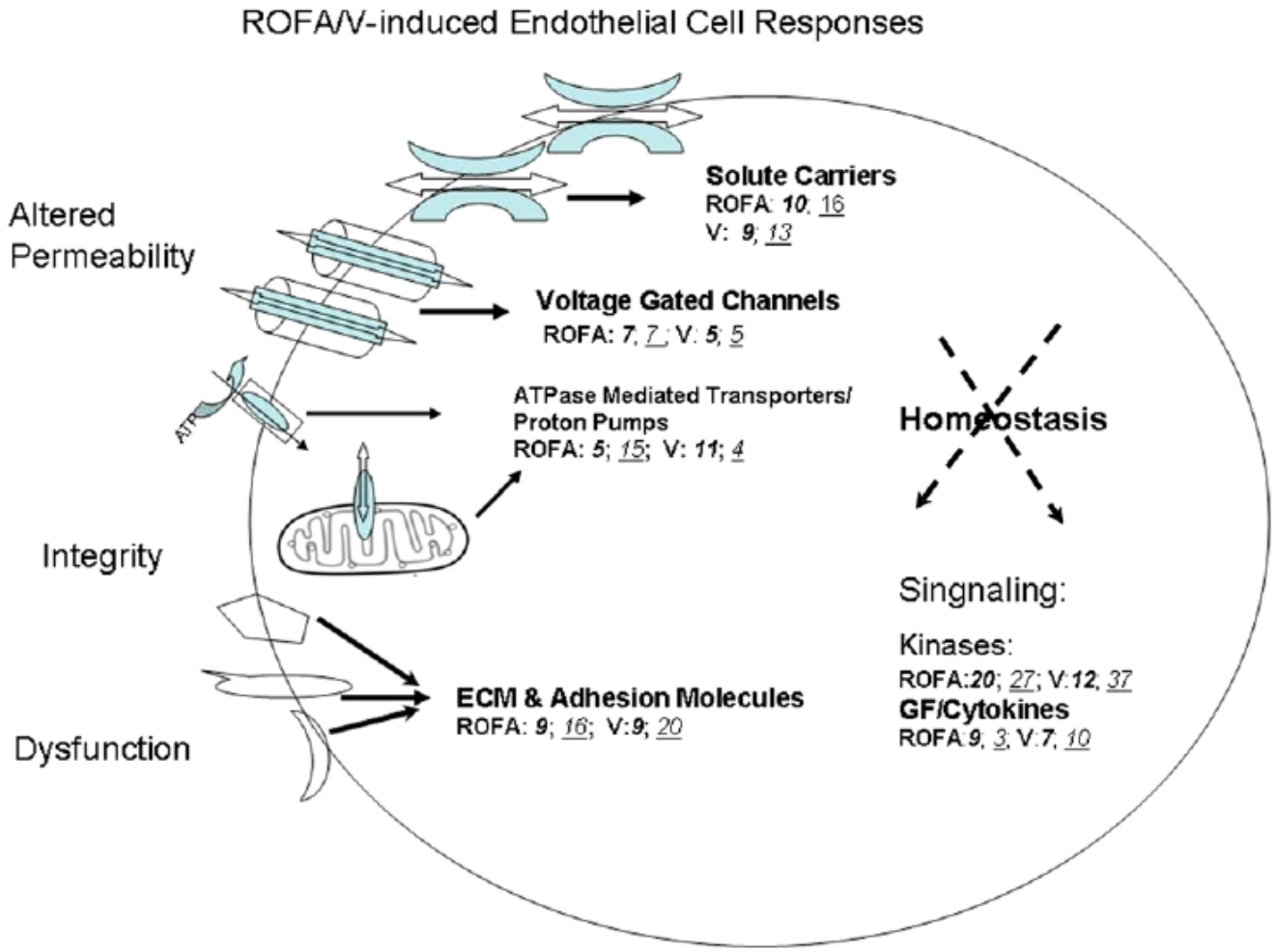

Fig. 2.

Schematic representation of role and potential relationship among differentially altered genes in elucidating toxic response in human vascular endothelial cells. The numbers indicates the number of genes each category that are altered (derived from the data presented in Tables 5–7) due to ROFA or V exposure. Induced genes are presented in **bold italics**, while suppressed genes are presented by italics with underline. ECM: extracellular matrix.

Table 1

Number of genes found differentially expressed in HUVEC on exposure to ROFA or V.

Group	Induced (>2-fold)	Suppressed (>2-fold)
ROFA	339	471
Vanadium	306	508
Common	119	226
Unique to ROFA	220	245
Unique to vanadium	187	282

Table 2

Distribution of differentially expressed genes into various gene families.

Functional Groups	ROFA		Vanadium		Common	
	Up	Down	Up	Down	Up	Down
Cytokines/growth factors (131*)	7	3	5	10	2	0
Cell adhesion molecules (53*)	3	0	2	4	0	2
Extracellular matrix (126*)	3	3	4	3	3	11
Signal transduction (855*)	50	48	34	59	27	51
Transporters (188*)	10	23	15	9	5	8
Kinases (256*)	14	5	6	15	6	22
Voltage dependent signaling (68*)	4	5	2	3	3	2

*Number of genes in the microarray representing each gene family.

Table 3

ROFA-induced acute differential gene expression profiles in HUVEC.

Accession #	Gene name	Fold change
Solute carrier proteins		
NM_001042	Solute carrier family 2 (facilitated glucose transporter), member 4	2.81
NM_003045	Solute carrier family 7 (cationic amino acid transporter), member 1	2.70
NM_002394	Solute carrier family 3 (dibasic and neutral amino acid transport), member 2	2.47
NM_000339	Solute carrier family 12 (Na/Cl transporters), member 3	2.42
J03810	Solute carrier family 2 (facilitated glucose transporter), member 2	2.31
NM_005072	Solute carrier family 12 (K/Cl transporters), member 4	2.22
NM_006672	Solute carrier family 22 (organic anion transporter), member 7	2.22
NM_006598	Solute carrier family 12 (K/Cl transporters), member 7	2.20
NM_003049	Solute carrier family 10 (Na/bile acid cotransporter family), member 1	−6.33
NM_001152	Solute carrier family 25 (adenine nucleotide translocator), member 5	−4.62
NM_005074	Solute carrier family 17 (sodium phosphate), member 1	−3.79
NM_004785	Solute carrier family 9 (Na/H exchanger)	−2.31
NM_007163	Solute carrier family 14 (urea transporter), member 2	−2.26
NM_003042	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1	−2.23
Z18956	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	−2.21
NM_004172	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	−2.09
NM_005698	Secretory carrier membrane protein 3	−2.06
NM_005628	Solute carrier family 1 (neutral amino acid transporter), member 5	−2.02
NM_005628	Solute carrier family 1 (neutral amino acid transporter), member 5	−2.01
ATPase mediated transport proteins		
U57971	ATPase, Ca++ transporting, plasma membrane 3	5.76
NM_001694	ATP6L	2.55
NM_001682	ATPase, Ca++ transporting, plasma membrane 1	−8.99
U11700	ATPase, Cu++ transporting, beta polypeptide (Wilson disease)	−6.06
NM_001677	ATPase, Na+/K+ transporting, beta 1 polypeptide	−5.27
NM_001691	ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha isoform 2	−4.64
NM_000702	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	−3.60
NM_001689	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c3	−3.52
NM_001685	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6	−3.50
X69908	ATP synthase, H+transporting, mitochondrial F0 complex, subunit c 2	−3.25
D00099	ATPase, Na+/K+ transporting, alpha 1 polypeptide	−2.40
NM_001676	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	−2.38
NM_001688	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	−2.04
NM_006356	ATP synthase, H+ transporting, mitochondrial F1F0, subunit d	−2.01
Voltage-gated channels		
NM_012285	K voltage-gated channel, subfamily H (eag-related), member 4	3.07
NM_012283	K voltage-gated channel, subfamily G, member 2	2.33
NM_005136	K voltage-gated channel, Isk-related family, member 2	2.27
NM_006078	Ca channel, voltage-dependent, gamma subunit 2	2.15
NM_012282	K voltage-gated channel, Isk-related family, member 1-like	−8.26

Accession #	Gene name	Fold change
NM_002236	K voltage-gated channel, subfamily F, member 1	−4.64
NM_000069	Ca channel, voltage-dependent, L type, alpha 1S subunit	−2.84
NM_005472	K voltage-gated channel, Isk-related family, member 3	−2.14
NM_000725	Ca channel, voltage-dependent, beta 3 subunit	−2.05
Kinases		
NM_006549	Ca /calmodulin-dependent protein kinase kinase 2 beta	11.12
NM_015978	Putative protein tyrosine kinase	8.14
NM_006282	Serine/threonine kinase 4	4.62
L41939	EphB2	4.22
AF078078	Growth arrest and DNA-damage-inducible, gamma	3.39
D17517	TYRO3 protein tyrosine kinase	3.20
L13616	PTK2 protein tyrosine kinase 2	3.06
L40636	EphB1	2.87
U40282	Integrin-linked kinase	2.77
NM_006257	Protein kinase C, theta	2.75
NM_003576	Serine/threonine kinase 24 (Ste20, yeast homolog)	2.55
NM_006259	Protein kinase, cGMP-dependent, type II	2.33
X66362	PCTAIRE protein kinase 3	2.25
NM_003684	MAP kinase-interacting serine/threonine kinase 1	2.18
U43142	Vascular endothelial growth factor C	−3.63
NM_004920	Apoptosis-associated tyrosine kinase	−2.24
U25265	Mitogen-activated protein kinase kinase 5	−2.21
NM_006575	Mitogen-activated protein kinase kinase kinase kinase 5	−2.18
Z29090	PIK3CA	−2.01
Cytokines, and growth factors		
NM_002620	Platelet factor-4 variant 1	5.22
NM_000598	Insulin-like growth factor binding protein 3	2.64
NM_001553	Insulin-like growth factor binding protein 7	2.36
NM_002573	PAF (acetylhydrolase, isoform Ib, gamma subunit)	2.27
X02811	PDGFB	2.12
D12614	Lymphotoxin alpha (TNF superfamily, member 1)	2.05
NM_003867	Fibroblast growth factor-17	2.02
NM_006654	Suc1-associated neurotrophic factor target	−3.05
M_004970	Insulin-like growth factor binding protein, acid labile subunit	−2.70
NM_003692	Transmembrane protein with EGF-like and two follistatin-like domains 1	−2.55
Cell adhesion molecules and extra cellular matrix		
X15880	Collagen, type VI, alpha 1	3.33
NM_007046	Elastin microfibril interface located protein	2.80
NM_001084	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	2.30
X15606	Intercellular adhesion molecule 2	2.97
D38163	Collagen, type XIX, alpha 1	2.30
NM_002162	Intercellular adhesion molecule 3 (Icam-3)	2.14

Accession #	Gene name	Fold change
X75308	Matrix metalloproteinase 13 (collagenase 3)	−2.50
X05610	Collagen, type IV, alpha 2	−2.48
L22548	Collagen, type XVIII, alpha 1	−2.42

Table 4

Vanadium-induced acute differential gene expression profiles in HUVEC.

Accession #	Gene name	Fold change
Solute carrier proteins		
NM_012434	Solute carrier family 17 (anion/sugar transporter), member 5	8.41
NM_007163	Solute carrier family 14 (urea transporter), member 2	2.70
NM_007256	Solute carrier family 21 (organic anion transporter), member 9	2.64
NM_005847	Solute carrier family 23 (nucleobase transporters), member 2	2.44
NM_001046	Solute carrier family 12 (Na/K/Cl transporters), member 2	2.22
NM_005075	Solute carrier family 21 (organic anion transporter), member 3	2.03
NM_004803	Organic cationic transporter-like 4	2.01
NM_003984	Solute carrier family 13 (Na-dependent dicarboxylate transporter), member 2	−3.72
NM_014437	Zinc/iron regulated transporter-like	−3.54
NM_001042	Solute carrier family 2 (facilitated glucose transporter), member 4	−2.89
NM_007105	Solute carrier family 22 (organic cation transporter), member 1-like antisense	−2.70
NM_016354	Solute carrier family 21 (organic anion transporter), member 12	−2.33
NM_006598	Solute carrier family 12 (potassium/chloride transporters), member 7	−2.25
U13173	Solute carrier family 15 (oligopeptide transporter), member 1	−2.22
NM_003459	Solute carrier family 30 (zinc transporter), member 3	−2.15
ATPase mediated transport proteins		
NM_001690	ATPase, H+ transporting, lysosomal (vacuolar proton pump), isoform 1	5.72
NM_001697	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	5.02
NM_006476	ATP synthase, H+ transporting, mitochondrial F1F0, subunit g	4.87
NM_001686	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	4.41
NM_005765	ATPase, H+ transporting, lysosomal (vacuolar proton pump)	3.90
NM_004889	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f 2	3.47
NM_001682	ATPase, Ca++ transporting, plasma membrane 1	2.23
NM_005173	ATPase, Ca++ transporting, ubiquitous	2.00
NM_001694	ATP6L	−2.44
Voltage-gated channels		
NM_004519	K voltage-gated channel, KQT-like subfamily, member 3	2.59
NM_006514	Na channel, voltage-gated, type X, alpha polypeptide	2.01
NM_002232	K voltage-gated channel, shaker-related subfamily, member 3	−7.36
NM_004976	K voltage-gated channel, Shaw-related subfamily, member 1	−2.77
NM_014405	Ca channel, voltage-dependent, gamma subunit 4	−2.16
Kinases		
NM_007271	Serine threonine protein kinase	6.97
NM_004717	Diacylglycerol kinase, iota	6.32
M15476	Plasminogen activator, urokinase	4.54
AB018261	Diacylglycerol kinase, beta (90kD)	4.22
NM_004635	Mitogen-activated protein kinase-activated protein kinase 3	3.82
NM_006254	Protein kinase C, delta	2.90
NM_006549	Calcium/calmodulin-dependent protein kinase kinase 2, beta	−6.63
M95712	V-raf murine sarcoma viral oncogene homolog B1	−5.66

Accession #	Gene name	Fold change
NM_001892	Casein kinase 1, alpha 1	−4.47
L05148	Zeta-chain (TCR) associated protein kinase (70 kD)	−4.44
M16591	Hemopoietic cell kinase	−4.14
Y10256	Mitogen-activated protein kinase kinase kinase 14	−4.13
Y07512	Protein kinase, cGMP-dependent, type I	−3.40
NM_006259	Protein kinase, cGMP-dependent, type II	−2.80
U62540	Anaplastic lymphoma kinase (Ki-1)	−2.77
NM_002730	Protein kinase, cAMP-dependent, catalytic, alpha	−2.61
NM_002742	Protein kinase C, mu	−2.59
X51602	Fms-related tyrosine kinase 1 (VEGF receptor)	−2.40
NM_012407	Protein kinase C, alpha binding protein	−2.13
NM_006257	Protein kinase C, theta	−2.04
NM_002741	Protein kinase C-like 1	−2.00
Cytokines, & growth factors		
NM_004469	Vascular endothelial growth factor D	5.25
NM_002195	Insulin-like 4 (placenta)	4.02
NM_005478	Insulin-like 5	2.78
NM_000300	Phospholipase A2, group IIA (platelets, synovial fluid)	2.04
M93718	Nitric oxide synthase 3 (endothelial cell)	2.05
NM_000407	Glycoprotein Ib (platelet), beta polypeptide	−3.45
NM_003597	TGFB inducible early growth response 2	−3.08
NM_003867	Fibroblast growth factor-17	−3.05
M62403	Insulin-like growth factor binding protein 4	−2.82
NM_001061	Thromboxane A synthase 1 (platelet, cytochrome P450, subfamily V)	−2.63
NM_005130	Heparin-binding growth factor binding protein	−2.51
NM_004740	TGFB1-induced anti-apoptotic factor-1	−2.37
NM_004590	Small inducible cytokine subfamily A (Cys-Cys), member 16	−2.27
NM_005542	Insulin induced gene 1	−2.23
M77227	Hepatocyte growth factor (hepapoietin A; scatter factor)	−2.11
Cell adhesion molecules andextra cellular matrix		
NM_000905	Neuropeptide Y	10.95
NM_003775	Endothelial differentiation, G-protein-coupled receptor 6	3.67
M62880	Integrin, beta 7	3.2
NM_003183	Disintegrin and metalloproteinase (TNF-a, converting enzyme)	2.24
U04810	Trophinin associated protein (tastin)	2.18
M74816	Clusterin (complement lysis inhibitor)	2.13
NM_001544	Intercellular adhesion molecule 4 (Icam – 4)	−9.77
NM_000887	Integrin, alpha X (antigen CD11C (p150), alpha polypeptide)	−4.33
AF031385	Cysteine-rich, angiogenic inducer, 61	−3.61
NM_002204	Integrin, alpha 3 (antigen CD49C, a3)	−2.68
NM_003637	Integrin, alpha 10	−2.65
NM_005010	Neuronal cell adhesion molecule	−2.44

Accession #	Gene name	Fold change
NM_005099	A disintegrin-like and metalloprotease (reprolysin type)	−2.01

Table 5

Genes found altered in HUVEC on acute exposure to both ROFA and Vanadium.

Accession #	Gene name	Fold change
Solute carrier proteins		
NM_004695	Solute carrier family 16 (monocarboxylic acid transporters), member 5	2.63
NM_003982	Solute carrier family 7 (cationic amino acid transporter), member 7	2.51
U03506	Solute carrier family 1 (glutamate transporter), member 1	−9.50
NM_001859	Solute carrier family 31 (copper transporters), member 1	−6.40
L14595	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	−3.52
NM_013309	Solute carrier family 30 (zinc transporter), member 4	−3.35
S70609	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9	−3.03
ATPase mediated transport proteins		
L20977	ATPase, Ca++ transporting, plasma membrane 2	4.85
NM_000544	ATP-binding cassette, subfamily B (MDR/TAP), member 3	4.01
NM_000703	ATPase, Na+/K+ transporting, alpha 3 polypeptide	2.42
NM_004047	ATP6F	−8.01
NM_001183	ATPase, H+ transporting, lysosomal (vacuolar proton pump), subunit 1	−7.02
NM_012069	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	−3.56
NM_005183	Calcium channel, voltage-dependent, alpha 1F subunit	12.95
Voltage-gated channels		
NM_006030	Ca channel, voltage-dependent, alpha 2/delta subunit 2	3.95
NM_003375	voltage-dependent anion channel 2	2.22
NM_000723	Ca channel, voltage-dependent, beta 1 subunit	−8.93
NM 004700	K voltage-gated channel, KQT-like subfamily, member 4	−2.06
Kinases		
NM_002740	protein kinase C, iota	5.54
U39657	Mitogen-activated protein kinase kinase 6	3.83
X16416	V-abl Abelson murine leukemia viral oncogene homolog 1	3.70
NM_003629	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)	3.67
X66365	Cyclin-dependent kinase 6	2.97
NM_006609	Mitogen-activated protein kinase kinase kinase 2	2.23
X85960	Neurotrophic tyrosine kinase, receptor, type 1	−8.62
NM_001347	Diacylglycerol kinase, theta (110kD)	−8.01
NM_004760	Serine/threonine kinase 17a (apoptosis-inducing)	−7.28
NM_001896	Casein kinase 2, alpha prime polypeptide	−6.70
NM_004383	C-src tyrosine kinase	−5.92
NM_001346	Diacylglycerol kinase, gamma (90kD)	−5.85
X66360	PCTAIRE protein kinase 2	−4.96
NM_001260	Cyclin-dependent kinase 8	−4.61
M30448	Casein kinase 2, beta polypeptide	−4.51
NM_001721	BMX non-receptor tyrosine kinase	−4.48
NM_004834	Mitogen-activated protein kinase kinase kinase kinase 4	−4.35
X70040	Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	−4.29
D25328	Phosphofructokinase, platelet	−3.82

Accession #	Gene name	Fold change
NM_005922	Mitogen-activated protein kinase kinase kinase 4	−3.61
X06318	Protein kinase C, beta 1	−2.90
NM_004103	Protein tyrosine kinase 2 beta	−2.75
NM_002732	Protein kinase, cAMP-dependent, catalytic, gamma	−2.57
U51004	Histidine triad nucleotide-binding protein	−2.43
U09564	SFRS protein kinase 1	−2.15
U09607	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	−2.10
X66363	PCTAIRE protein kinase 1	−2.08
NM_005975	PTK6 protein tyrosine kinase 6	−2.07
Cytokines, & growth factors		
AB011541	EGF-like-domain, multiple 4	7.67
M65062	Insulin-like growth factor binding protein 5	2.83
Cell adhesion molecules and extra cellular matrix		
NM_004995	Matrix metalloproteinase 14 (membrane-inserted)	6.04
NM_001816	Carcinoembryonic antigen-related cell adhesion molecule 8	3.79
NM_002403	Microfibrillar-associated protein 2	2.59
NM_003734	Amine oxidase, copper containing 3 (vascular adhesion protein 1)	−11.67
NM_005200	Cell matrix adhesion regulator	−11.06
X05231	Matrix metalloproteinase 1 (MMP-1)	−10.77
NM_005203	Collagen, type XIII, alpha 1	−9.65
NM_000088	Collagen, type I, alpha 1	−8.80
M24795	CD36 antigen	−8.17
NM_000442	Platelet/endothelial cell adhesion molecule (CD31 antigen)	−6.03
NM_004142	Matrix metalloproteinase-like 1	−3.32
D21337	Collagen, type IV, alpha 6	−3.29
X03124	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	−3.06
NM_003076	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	−2.94
NM_000093	Collagen, type V, alpha 1	−2.80
NM_000918	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide protein	−2.41

Table 6

Activation profiles for transcription factor in HUVEC on acute exposure to ROFA or V.

Transcription factor	Control	ROFA	Vanadium
AP-1	21.9*	258.4	450.7
AP-2	27.0	496.7	759
BRN-3	44.1	321.9	419.3
CDP	221.7	338.7	568.2
c-Myb	387.3	832.7	746.8
Ets-1/PEA3	131.8	244.4	501.7
GATA	185.1	328.6	249.4
HNF-4	317.4	914.5	971.4
IRF-1	205.3	386.9	477.3
MEF-1	158.3	383.4	399.6
NF-E2	85	162.5	420.2
p53	119.9	125.6	240.8
Pax-5	1094.8	1296.5	1199.7
Pit 1	292.0	439.1	503.8
PRE	320.7	324.7	405.3
Smad/SBE	1212.2	1378.7	1190.8
Stat5	112.6	429.5	134.9
Stat5/Stat6	105.7	478.3	280.7
USF-1	146.7	643.5	679.0

*Mean average spot density (absorbance value) value for each TF from three blots.

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Table 7

Computationally identified transcription factor binding sites in genes found induced in HUVEC by ROFA or V.

		AP-1	Brn-2	c-Ets	CDP	HNF-4	NF-E2	STAT4/5	USF-1
ROFA	Platelet Factor-4	+		+			+		
ROFA	PDGF-B			++			+	+	++
ROFA	TNF-beta	++							++
ROFA	ICAM-2								++++
ROFA	PAF			+					
ROFA	TNFSF8	+++							++
ROFA	ICAM-3				+				+
ROFA	Insulin like growth factor-5		+	++					+++
ROFA	STK4-MST1	+		+	++				
ROFA	PKC-theta		++		+				+
ROFA	K-voltage-gated channel	+++		++++					
ROFA	MNK1			+					
ROFA	Ca-ATPase				+				++++
ROFA	CAMKK	+++		+++					+++++
ROFA	MAPKAP3	++		++++	++++		+		++++
V	INSL5		+	++					+++
V	PKC-delta	++		++++++++					+++
V	ATP Synthase-H+	+++	+			+			
V	Solute Carrier protein14					+	+	+	+
V	Organinic-cationic-trasport				++			++	+++++
V	K-Voltage-gated channel	+++				+		+	
V	Na-Voltage gated channel	+++++	+	+++++				+	
V	Pro-Urokinase	+							++
V	DAGK	++	+	++				++	++++
Common	MEGF8	+++		+++++					
Common	VGF-I	+++		+++++				++	++++++++++++
Common	PKC-iota	++++		+					
Common	MKK-6	+++			+			+	+++++++++
Common	Ca-ATPase-2, PMCA-2	+							+++
Common	Na/KATPase	+		+					

	AP-1	Brn-2	c-Ets	CDP	HNF-4	NF-E2	STAT4/5	USF-1

[+] Represent one binding site for TF on the gene.