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Systems Analysis of Oxidant Stress in the Vasculature

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Abstract

Systems biology and network analysis are emerging as valuable tools for the discovery of novel relationships, the identification of key regulatory factors, and the prediction of phenotypic changes in complex biological systems. Redox homeostasis in the vasculature is maintained by an intricate balance between oxidant-generating and antioxidant systems. When these systems are perturbed, conditions are permissive for oxidant stress, which, in turn, promotes vascular dysfunction and structural remodeling. Owing to the number of elements involved in redox regulation and the different vascular pathophenotypes associated with oxidant stress, vascular oxidant stress represents an ideal system to study by network analysis. Networks offer a method to organize experimentally derived factors, including proteins, metabolites, and DNA, that are represented as nodes into an unbiased comprehensive platform for study. Through analysis of the network it is possible to determine essential or regulatory nodes, identify previously unknown connections between nodes, and locate modules, which are groups of nodes located within the same neighborhood that function together and have implications for phenotype. Investigators have only recently begun to construct oxidant stress-related networks to examine vascular structure and function; however, these early studies have provided mechanistic insight to further our understanding of this complicated biological system.

Keywords

Systems biology; networks; interactome; oxidant stress; antioxidants

Introduction

Systems biology and its related disciplines have witnessed a tremendous growth in the past decade, owing, in part, to the development and implementation of high-throughput analytical methodologies that yield large datasets. These complex datasets are typically mined using a reductionist approach to identify a few differentially expressed genes or proteins associated with a phenotype of interest while ignoring large parts of the data. The advantage of applying systems biology methods to these datasets is the ability to consider simultaneously all (or large numbers of) genes, proteins, and/or metabolites and their interactions through networks. Analysis of networks is not limited by the structural complexity of the model studied (e.g., cell, tissue, or organism) or the experimental conditions. Moreover, networks allow for the discovery of new interactions among

components, the identification of critical regulatory points within a biological system, and the prediction of phenotypic changes.

Networks that model biological systems can simplify complex relationships. Thus, in considering oxidant stress and vascular function, a network may be constructed by integrating components from endothelial and vascular smooth muscle cells, fibroblasts, and circulating cell types, together with vasoactive peptides, hormones, and other biological mediators that regulate levels of reactive oxygen species (ROS) and their derivatives. Interactions between components (i.e., gene-protein; protein-protein; protein-metabolite) and functional links such as between genes or genetic polymorphisms, gene transcripts, and gene products are utilized to create an integrated network. The network may then be analyzed to provide novel insights about a particular cellular function (ROS generation or reduction in vascular cells), a physiological process (vascular reactivity), a pathophysiological response (oxidant stress and vascular dysfunction), or a disease state (hypertension or atherosclerosis). The use of systems biology methods to understand biological processes as they relate to human disease has been the subject of many reviews in the past few years (1–5). Here, we discuss the role of oxidant stress in the vasculature, summarize some basic considerations regarding the principles of systems biology and networks, and examine how network analysis has been used to date to further our knowledge of the role of oxidant stress in modulating vascular pathophysiology.

Oxidant Stress and Vascular Function

In vascular cells ROS function as signaling molecules to facilitate basal homeostatic and metabolic processes within a balanced redox environment. When ROS are generated in excess and/or there is a concomitant decrease in the capacity to reduce ROS through enzymatic and small molecule antioxidant systems, the redox milieu is perturbed leading to oxidant stress conditions. When oxidant stress is present, ROS levels have exceeded the threshold required for ambient cell signaling and ROS may oxidatively modify or damage proteins, lipids, and DNA with potentially deleterious consequences for vascular structure and function (6–13).

The complexity of the vascular oxidant-antioxidant system is demonstrated by the array of enzymatic and non-enzymatic factors that have evolved to synthesize as well as metabolize cellular ROS (Table 1) (6–13). The role of these oxidant-antioxidant systems in maintaining normal cellular function and the consequences of disruption of ROS handling that results in vascular dysfunction have been the subject of many recent reviews (6–13).

While it is important to note that increases in ambient ROS levels often have deleterious effects on vascular cell function, in select cases, increased levels of oxidants activate redox switches that direct cellular protein function. In fact, oxidative posttranslational modification of reactive cysteinyl thiols has been shown to regulate protein function in response to environmental fluctuations in the cellular redox state (Fig. 1). These reactive cysteines may be modified by oxidation as well as glutathionylation or nitrosylation. For example, under conditions of increased oxidant stress, Cys122, which resides in the nitric oxide-sensing region of the β -subunit of soluble guanylyl cyclase, is oxidized to cysteinyl sulfinic acid,

sulfonic acid, and new disulfide bonds are formed to neutralize the sensitivity of the enzyme for activation by nitric oxide (14). Similarly, oxidation of a cluster of reactive cysteine residues (Cys402-Cys405) in the nitric oxide synthase activating region of the endothelin B receptor decreases nitric oxide production by limiting its synthesis (15). Through this and other mechanisms, increased ROS levels limit bioavailable nitric oxide to promote endothelial dysfunction, inflammation, and thrombosis and identify these reactive cysteinyl thiols as key regulatory redox switches that regulate the activation or function of a variety of enzymes, receptors, and transcription factors in response to alterations in cellular oxidant flux (15–25). These molecular signals, in turn, are important for vascular functional responses, such as vasodilation and Ca^{2+} handling, and modify phenotypic responses by regulating cell growth, apoptosis, senescence, and inflammation. Another equally important aspect of oxidant imbalance that is often overlooked is the detrimental effects of reductive stress, which may diminish growth factor mediated signaling, mitochondrial function, and subsequent cell growth and survival, in part, by lessening necessary disulfide bond formation or preventing the oxidative inactivation of cysteines in phosphatases (26–28). Reductive stress has also implicated in the pathogenesis of protein aggregation cardiomyopathy (29).

When considered *in toto*, the causes and consequences of oxidant stress for the vascular pathophenotype are substantial. The cellular components and signaling cascades that regulate oxidant stress are multifactorial; subject to genetic and posttranslational regulation, spatial, and temporal constraints; and operate on a dynamic continuum. The majority of studies that have examined oxidant stress in the vasculature have focused on select aspects of this relationship as opposed to evaluating the entire system within a broader context that considers all related aspects of oxidant stress and vascular function. This shortcoming highlights the necessity of employing a systems biology approach to understand fully the complexities of how vascular oxidant stress modulates phenotypic changes in blood vessels.

The Interactome and Network Construction

To simplify complex systems such as vascular oxidant stress, systems biology organizes the relevant molecular and macromolecular components into a rational framework, or network, for further analysis and discovery. The network takes shape as the individual components are arranged based on their known interactions, and is referred to as the interactome. The size or magnitude of the interactome is variable and is based on the included components. As an example, the human interactome is comprised of approximately 25,000 protein-coding genes, 1,000 metabolites, and an expanding list of functional RNAs and posttranslationally modified proteins. This catalog of components results in up to 100,000 possible components with an exponentially greater number of interactions (1, 12). It is, therefore, readily understandable that following a singularly focused experimental strategy would very likely overlook many of the potential relationships that are identified through network analysis.

Biologically relevant components that are used to construct a network may be found through curation from the literature, computational databases, or high throughput –omics data. Curation is a widely employed methodology; however, the results are limited by the search parameters selected and are dependent upon prior publications. There is also an inherent bias

introduced by the lack of equal publication priority for negative results. Computational prediction methods rely on indirect data such as sequence similarities. This method may generate large networks, although they are often considered to be intrinsically imperfect owing to the use of orthogonal data. Components identified from high-throughput –omics-based methods that consider entire genomes, proteomes, or metabolomes are favored as they are considered to be robust and unbiased (2, 4). While difficult to obtain in the past, these datasets are increasingly available as a result of user-friendly and less costly platforms, as well as public access to archived information.

Network modeling has also been advanced by the availability of online interaction database resources that provide information to detail how individual components that will be incorporated into a network are related (1, 4, 12). Protein-protein interactions have been curated in several databases, including the Biological and General Repository for Interaction Datasets (BioGRID) and the Human Protein Reference Database (HPRD) [1]. Databases that detail regulatory relationships pertaining to protein posttranslational modifications (e.g., phosphorylation, oxidation, acetylation, etc.) with activating or inhibitory function are in their nascent stages. The exception is for phosphorylation modifications, which are provided in online databases such as PhosphoELM, phosphorylation site database (PHOSIDA), or Phosphosite as well as the CBS prediction database. Biochemical networks defining relationships among metabolic components may be identified from sources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Biochemical Genetic and Genomics (BIGG) knowledgebase, or recently published compendiums of human metabolic and transport pathways (1).

Genomic regulatory databases, such as the Universal Protein Binding Microarray Resource for Oligonucleotide Binding Evaluation (UniPROBE) and JASPAR databases, are typically less comprehensive than protein-protein interaction databases but continue to incorporate information from chromatin immunoprecipitation-microarray or –sequencing studies (1, 12). Recently the Encyclopedia of DNA Elements (ENCODE) project published a series of studies that have begun to validate transcription factor-DNA interactions by correlating DNase sensitivity sites with DNA transcription factor motifs to identify active transcription factor sites within a given cell (30). This assumption is based on the premise that actively transcribed chromatin has an open conformation and is accessible to DNase I digestion. Additional information can be gathered from databases that compile genome-wide association studies (GWAS) or studies on single nucleotide polymorphisms (SNPs), although the actual functional consequences of most of these sequence variations are not well understood. MicroRNAs are another mechanism of genomic regulation, and there are an increasing number of databases that use sequence alignment prediction algorithms to identify putative targets, including TargetScan, miRBase, PicTar, and miRanda, or catalog experimentally verified interactions between microRNAs and targets in databases such as miRecords (12, 31).

When using these databases to extract information to create a network, several caveats exist. The first is that many databases are incomplete, and predicted interactions may require confirmation by traditional methods. Similarly, database information about the functional relevance of epigenetic or other posttranslational modifications is limited. Current methods

to predict microRNA targets may also yield false positive results, indicating a need to expand databases with validated microRNA targets. Thus, when constructing a network, it is important to recognize the inherent limitations associated with the data input and to modify this input as newer information becomes available.

Network Characteristics

Once the relevant components for the network have been identified, they are mapped based on known interactions to create the network structure: each component is a node, its connection with another node is referred to as an edge or link, and nodes that are connected to many other components are referred to as hubs (Fig. 2). Analysis of biological networks reveals that they tend to be 'scale-free,' that is, most nodes have few connections and only a few nodes are hubs with many connections. In these scale-free systems, the probability of interaction between nodes is unlikely to occur by chance alone and suggests that functional relationships exist between some nodes (1, 2). Interestingly, genes essential for development tend to encode hub proteins that reside at the center of the network while non-essential disease-related genes do not encode hubs, are cell type- or organ-specific, and frequently reside at the periphery of a network (1, 2, 4). Perturbation of an essential hub protein disrupts the network significantly and is less likely to occur or persist in nature, possibly due to negative selection, such as embryonic lethality and reduced fitness. Network architecture may also explain and predict phenotype. Although most nodes are separated by a limited number of edges, it is evident that perturbation of a single node may have repercussions throughout the network by affecting other connected nodes and their interaction partners (1, 5).

Biological networks include modules, which are groups of nodes that are located within the same local area or neighborhood, display a high degree of clustering, and have a high degree of connectedness between the nodes (Fig. 2) (1, 4). These topological modules or sub-networks are identified by unbiased clustering algorithms. They may be characterized further as functional modules in which components within the module share a similar or related function, or disease modules in which disease-related genes tend to interact with other like genes to confer the same pathophenotype on a cell, tissue, or organism. Nodes located within a disease module may also overlap with other modules thereby exposing novel relationships between disease-related and non-disease-related factors, or between different (canonical) diseases (1, 2). As data are acquired to gauge the strength and directionality of interactions between nodes within modules, analysis of flux through the network will prove informative by uncovering key regulatory points and highlighting unanticipated consequences of perturbing nodes (31).

Network Analysis of Oxidant Stress in the Vasculature

Using these principles, investigators have begun to construct networks to investigate factors that mediate the effects of oxidant stress on vascular structure and function. In the following selected illustrative studies, networks were used to probe interactions between ROS and other signaling molecules to identify novel mechanistic or regulatory relationships between oxidant stress, ROS-related signaling pathways, and cardiopulmonary vascular phenotype.

The exposure of non-diseased cardiovascular or pulmonary vascular cells to tobacco smoke is known to increase oxidant stress. To examine key signaling molecules involved in this response, a network was constructed that incorporated genes and proteins related to hypoxia, oxidant stress, endoplasmic reticulum (ER) stress, shear stress, and xenobiotic stress as determined from published studies. Next, to enhance the biological relevance of the network, reverse causal reasoning was applied to four transcriptomic data sets identified from experiments performed using cardiopulmonary cells exposed to stress conditions that were available through a public access database. The datasets selected were from experiments performed in mouse whole lung exposed to hyperoxia, mouse RAW 267.4 cells exposed to hypochlorous acid, human aortic endothelial cells exposed to oxidized phospholipids, and human lung microvascular endothelial cells exposed to hypoxia. Reverse causal reasoning is a method that is utilized to identify upstream biological mediators likely to regulate and thereby explain the observed (i.e., downstream) changes in expression. This information may then be used to validate an existing network and/or find additional nodes to add to a network. In this stress network example, reverse causal reasoning pointed to a role for NFkB activation in 3 of the 4 data sets analyzed. This proved to be a confirmatory (validation) finding as the stress network based on data mining already included NFkB activation. The dataset information was also able to identify additional nodes that were subsequently added to the network. The final stress network comprised 720 nodes with 1,280 edges (Fig. 3) (32).

To validate the cell stress network, microarray data obtained from wild-type mice and mice deficient in the antioxidant stress response transcription factor (erythroid-derived) nuclear factor like-2 (NFE2L2 or NRF2) exposed to cigarette smoke for 24 hours were examined. Tobacco smoke is known to induce cell stress, however, responses to tobacco smoke were not used to construct the network and, therefore, mapping data obtained from the wild-type and NRF2-deficient mice exposed to tobacco smoke to the network did offer novel insight into the role of NRF2 in modulating cell stress. Signaling pathways identified in the stress network could explain 88% and 80% of the mRNA expression changes in wild-type and NRF2 knockout mice exposed to tobacco smoke, respectively. Further analysis revealed that ~46% of all of the observed mRNA changes in wild-type mice exposed to tobacco smoke could also be explained by NRF2-mediated activation of oxidant stress-related signaling pathways, but that concomitant activation of ER stress occurred in a NRF2-independent manner (32). Activation of NRF2 is regulated primarily by oxidative modification of key cysteine residues in Keap1, its inhibitor, that target Keap1 for degradation and allow for nuclear localization of NRF2 and activation of its transcriptional program; however, other forms of NRF2 activation that may be less reliant on oxidant stress, have been described recently (33). Nonetheless, the findings that NRF2 as well as other stress-activated, redox-dependent transcription factors, such as KRAS and AP-1, contribute to the tobacco-induced stress response implicate ROS and antioxidant imbalance in these pathways.

Oxidant stress is also known to regulate vascular cell proliferation and viability. To begin to evaluate mechanisms that influence pulmonary cell proliferation, an integrated network that contained 848 nodes and 1,597 links was developed. This network incorporated elements that are ROS-related or perturbed by oxidant stress, including cell cycle, growth factors, intra- and extracellular signaling, cell-cell interactions, and epigenetics. To structure this

network, components were organized in blocks around these key areas of biology with cell cycle forming the central block that connected to growth factors, cell interaction, epigenetics and intra- and extracellular signaling modules. The network was verified using four different microarray datasets related to cell proliferation. Preliminary analyses confirmed the validity of the network by demonstrating across all test datasets that the pro-proliferative factors E2F1 and MYC were increased and the negative regulators of cell proliferation RB1 and CDKN1A were decreased. When reverse causal reasoning was applied, however, this methodology was only able to predict changes in ~45% of the network nodes indicating that the network was incomplete. This finding is not surprising when one considers that cell proliferation is regulated, in part, via non-transcriptional mechanisms, such as posttranslational modification (e.g., phosphorylation, oxidation, etc.), which are typically not revealed by microarray studies and are not accounted for fully in the network (34).

When oxidant stress is overwhelming, cells may undergo apoptosis, necrosis, autophagy, or senescence as a terminal fate. The role of ROS in the transition to these pathogenic states was investigated using a network comprised of 1,052 nodes and 1,538 edges that contained 34 subnetworks or modules describing discrete mechanisms affecting cell fate. The network was constructed to apply to processes related to pulmonary function and the nodes included in the network were derived from relevant proteins, gene transcripts, phosphorylation status, and protein activities as well as biological processes (GO terms), chemicals and small molecules identified through curation of the literature. When possible, connections or edges between nodes were defined as causal nodes when a cause-effect relationship was determined. In some cases, this information was gathered from published data that did not involve studies in lung or cardiovascular tissue, especially with regard to protein activity assays, protein-protein interactions, and protein kinase targets studies, as these findings were often generated in other cell types or cell-free systems. The network was refined through reverse causal reasoning involving the use of 4 transcriptomic data sets, two that analyzed DNA damage and two that analyzed senescence. Interrogation of the senescence module was performed using transcriptomic profiling data from human lung fibroblasts expressing HRAS G12V, which is an accepted model of senescence induction. This analysis predicted changes in expression of mediators of cellular senescence, including decreased expression of cell cycle activators (CCND1 and E2F family members), increased expression of cell cycle inhibitors (CDKN1A, CDKN2a, and RB1), and induction of secretory-associated senescence proteins through the activation of NFkB and CEBPB signaling, thereby linking senescence with inflammation (35). Others studies that interrogated senescence-related networks identified CDKN1A as a mediator of mitochondrial dysfunction and increased ROS production through a signaling pathway that culminates in TGF- β activation (36). This study used stochastic modeling in addition to experimentally-derived data to confirm the feedback mechanisms by which ROS promotes the DNA damage response that continues CDKN1A-mediated senescence and the role for CDKN1A in maintaining TGF- β activation. Thus, although this study used a simpler model, the findings from this work provide independent validation for a key role of CDKN1A in senescence pathways. Proteomics has also been used to construct an autophagy protein-protein interaction network consisting of 409 nodes with 751 interactions or edges. This network was constructed experimentally by using as bait a set of thirty-two human proteins known to be linked to autophagy or vesicular

trafficking to capture other cellular proteins. To accomplish this, bait proteins were hemagglutinin-tagged and immunoprecipitation assays were employed to pull down interacting proteins. Proteomics was then used to identify high-confidence interacting proteins, a subset of which was used as secondary baits to validate the network. This network demonstrated high interconnectivity between signaling modules with 22 cross-module links; numerous interactions between modules with ATG8 family members suggested that these proteins have a regulatory role in autophagy. The network also identified new components related to ATG8 conjugation, vesicle elongation, and autophagosome assembly. This network provides a platform for further analysis of autophagy under other stress conditions as well as the functional roles and interactions of the component proteins (37).

Oxidant stress has been implicated in the vascular dysfunction associated with diabetes mellitus and cardiovascular disease, and studies have attempted to identify causal mediators through network analysis. For example, one study examined non-atherosclerotic arterial vessels harvested from 10 diabetic and 11 age-matched non-diabetic men at the time of coronary artery bypass grafting surgery. Differentially expressed genes (477 downregulated, 169 upregulated) were integrated with the human interactome network to identify 5 highly overlapping modules. The largest of these contained 74 nodes and identified INSR, CDC42, IRS2, SMAD4, and MMP2 as hubs. By comparison of findings between diabetic and non-diabetic vessels, it was evident that perturbation of nodes connected to these hubs would disrupt insulin receptor signaling, TGF- β signaling, cell growth, and extracellular matrix remodeling, all of which have been implicated in diabetic vascular dysfunction and remodeling (38). Other network-based studies have found that oxidant stress is a common molecular mechanism that links diabetes mellitus with hypertension and cardiovascular disease. Here, integrated analysis of candidate disease genes and a protein-protein interaction network identified 42 co-regulated components that mapped to 19 different signaling pathways. Further analysis identified 2 common signaling pathways involving insulin signaling, oxidant stress, and inflammation regulated by the E1A binding protein p300, which mediates cell growth and division, and peroxisome proliferator-activated receptor signaling, which link oxidant stress with diabetes mellitus and hypertension (39).

Network analysis has been applied to identify key regulators in essential hypertension. In this case, a protein-protein interaction network that contained 535 nodes and 2,572 edges was constructed using components identified by literature curation starting from 69 candidate genes associated with essential hypertension that were converted to seed proteins; however, the majority of the nodes in the network had relatively few connections. Within the network, NOS3 was identified as a node with high betweenness centrality, which is a measure of how often nodes occur on the shortest paths between other nodes and indicates a regulatory role for the node in the network. This makes biological sense as neighbors of NOS3 in the network were related to the SIRT1 pathway, antioxidants, inflammation, the renin-angiotensin system, and calcium signaling, all pathways involved in essential hypertension (40). One could also conclude from this network that when NOS3 is perturbed, the effect on vascular cell phenotype will extend beyond hypertension.

Atherosclerosis is a complex multifactorial disease with a predilection for the coronary arteries and often associated with endothelial injury and dysfunction. Network analysis has been performed to identify differences between coronary artery and non-coronary endothelium that may explain this phenomenon. Microarrays performed on endothelial cells isolated from coronary and non-coronary artery endothelium found that 1,297 of 10,798 assayed genes were differentially expressed between the two vascular beds. Weighted gene coexpression networks found 19 modules, 12 of which were related to circulatory bed classification. These modules were enriched for genes related to ER stress and oxidant stress/redox homeostasis. Further comparative analysis demonstrated that genes involved in ER stress pathways mapped to endothelium located in atherosusceptible zones in the vasculature. It was also found that the antioxidant genes, peroxiredoxin 2, thioredoxin, and the antioxidant-related transcription factor NRF2, included in the oxidant stress/redox homeostasis module were expressed to a lesser extent in the coronary artery endothelium as compared to non-coronary vascular beds. *In vitro* studies performed in endothelial cells confirmed that there was an increase in ROS and oxidant stress in coronary endothelial cells as compared to noncoronary endothelium consistent with the predictions found through analysis of the network (41).

The biology of another oxidant stress-related vascular disease, abdominal aortic aneurysm formation, is characterized by vascular smooth muscle cell apoptosis, extracellular matrix degradation, inflammation, and increased ROS. Using human aortic aneurysm specimens explanted at the time of surgery, a microRNA (miRNA) microarray identified 8 differentially expressed miRNAs compared to non-diseased infrarenal aortic tissue. Putative targets of these miRNAs were identified using a computational prediction database, and miRNA-target gene interactions were used to construct a network. The application of Gene Ontology terminology to the targets revealed that apoptosis and T cell activation were the most significantly represented biological functions. Network analysis identified the apoptosis mediators, tumor necrosis factor receptors TNFRSF8 and TNFRSF10B, as well as tumor protein p53-inducible nuclear protein 1 (TP53INP1), which are increased by oxidant stress and are predicted miRNA targets, as related to aneurysm formation. Similarly, the network identified the T cell-related molecules, CD28, CD86, and ICOS, which are targets of several miRNAs identified by the microarray. These T cell-related molecules were also shown to be elevated in the aortic wall of aneurysms (42).

Integrated networks may be used to identify downstream functionally related targets of candidate miRNAs involved in disease regulation. For example, a bioinformatics approach was employed to construct a pulmonary hypertension network that incorporated components related to hypoxia, TGF/BMP signaling, and inflammation, which are important regulatory mechanisms in pulmonary hypertension. The network was then mapped to a consolidated interactome to demonstrate functional interconnectivity among the target genes. Using a miRNA target prediction database and hypergeometric analysis, 29 microRNAs were identified that were highly likely to regulate the network. Of these, miRNA-21 was selected for further analysis and validation. This miRNA was shown to regulate bone morphogenetic protein receptor-2 expression as well as RhoB expression and Rho kinase activity, all key mediators of pulmonary vascular reactivity. Moreover, miRNA-21 expression was induced

by BMP signaling, hypoxia, and inflammation (IL-6), thereby confirming its integrative role in pulmonary hypertension pathobiology (43).

Therapeutic potential of network biology

The application of network biology to human disease has the potential to provide a more thorough understanding of pathobiology and contribute to the development of new therapeutic treatments of disease. For example, in cancer medicine, recent advances in the understanding of growth factor pathways and angiogenic signaling have been exploited through the view of pharmacogenomics and systems biology to develop novel therapies and usher in the era of personalized cancer medicine (44, 45). In transplant cardiology, scrutiny of -omics studies have helped to define a transcriptomic profile that allows for identification of rejection episodes in heart transplant patients, although this screening process has not yet achieved widespread clinical use. Nonetheless, application of this method may decrease the frequency of invasive testing, decrease the dose or number of anti-rejection medications required, and potentially improve patient survival through early noninvasive screening measures (46). Although an application of these findings may lead to improved patient care and outcomes, findings from these and other like studies require further verification in appropriate patient cohorts.

Conclusion

Network analysis is emerging as valuable tool for discovery in complex biological systems, such as oxidant stress in the systemic and pulmonary circulations. Historically, studies that have investigated the adverse effects of oxidant stress have examined a single molecule or signaling pathway and related it to a particular vascular phenotype. Owing to the large number of factors that regulate redox balance and the diversity of effects of oxidant stress on vascular structure and function, it is evident that this approach is incomplete. The utility of constructing a network for expansive fields such as oxidant stress is that it allows for the unbiased simultaneous consideration of any and all possibly related factors to facilitate the identification of novel interactions between components. Furthermore, network analysis provides insight into the consequences of disrupting a biological system, identifying disease-related components, or predicting unexpected effects from pharmacological interventions or genetic manipulations. With the increasing availability of large datasets and the continuous expansion of online databases, it is likely that in the near future there will be a paradigm shift of focus to network analysis as the primary approach to understanding disease processes.

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Figure 1. Oxidant stress and protein thiol redox switches

Vascular cells possess a number of enzymatic systems that generate reactive oxygen species, which, in turn, can modulate protein function through oxidative posttranslational modification of susceptible cysteinyl thiols. In vascular cells, oxidants are generated by the NADPH oxidases, xanthine oxidase, lipoxygenases, and cyclooxygenase. The endothelial isoform of nitric oxide synthase (eNOS) releases superoxide when it is “uncoupled” and reactive oxygen species may be released by mitochondria as part of normal cellular respiration (above dotted line). When high concentrations of superoxide or hydrogen peroxide (H_2O_2) are present near a protein cysteine residue (C) with a susceptible cysteinyl thiol ($-\text{SH}$), this cysteinyl thiol may be oxidized to higher order species, including sulfinic acid ($-\text{SO}_2\text{H}$) or sulfonic acid ($-\text{SO}_3\text{H}$) or disulfide bond ($-\text{S}-\text{S}-$) formation may occur. When this oxidation occurs, the protein is rendered inactive. The modification may be, in part, reversible and when the oxidized thiols are reduced, the protein then becomes active. This redox switch mechanism has been shown to regulate function of the endothelin B receptor and soluble guanylyl cyclase under oxidant stress conditions in vascular cells (14,15). O_2 , oxygen, SOD, superoxide dismutase.

Figure 2. Introduction to network terminology and network analysis

A) Networks are comprised of components that are referred to as nodes, the connection between nodes is an edge or link, and nodes that are highly connected to other components are referred to as hubs. Within networks, essential components tend to exist as hubs and reside at the center of the network, while single node disease-related factors are more likely to occur at the periphery. **B)** Modules are groups of related nodes that reside within a network and are located within the same area or neighborhood, display a high degree of clustering, and have a high degree of connectedness between nodes. Modules may be defined by their geographic location and propensity of nodes to cluster together (topological module), by sharing a common function that leads to node clustering (functional module), or by grouping in a manner such that perturbation of any of the nodes results in a similar disease phenotype (disease module). **C)** Network construction and analysis is a multistep process that involves the identification of seed genes, proteins, metabolites, or posttranslational modifications to be included in the network. These putative nodes are identified from literature curation, online databases, and public repositories of -omics studies. The nodes are used to construct an interactome and this network is explored for modules of related nodes. The modules are then used to identify previously unrecognized signaling pathways of interest as well as other components that interact with the network. From this information, the pathway may be validated using experimental methodology or used to predict other interactions or responses to treatments. Modified from (1) with permission.

Figure 3. Cellular stress network

A) A network comprised of genes and proteins related to hypoxia, oxidant stress, endoplasmic reticulum (ER) stress, shear stress, and xenobiotic stress was constructed to examine cardiovascular and pulmonary vascular cell responses to stress. The resulting network contained 720 nodes and 1,280 edges. The network was reconstructed using publically available data provided by the authors using Cytoscape 2.8.3 (27). **B)** The NRF2 (NFE2L2) module was identified in the cellular stress network. The module highlights the interactions between NRF2 and other signaling components, such as KEAP1, within the network. When examining this module, it is apparent that NRF2 interacts with factors that influence a diverse number of oxidant stress-related cellular processes, including apoptosis (CASP1 and CASP3), metabolism (PPARG), endoplasmic reticulum stress (ATF4), and cell signaling (JUN, CREBBP, MAPK). Modified from (27) with permission. NFE2L2, nuclear factor (erythroid-derived 2)-like 2; CASP1, caspase 1; CASP3, caspase 3; EIF2AK3, eukaryotic translation initiation factor 2- α kinase 3; COPS7A, COP9 constitutive photomorphogenic homolog subunit 7A; CHD6, chromodomain helicase DNA binding protein 6; PPARG, peroxisome proliferator-activated receptor- γ ; PMF1, polyamine-modulated factor 1; KEAP1, Kelch-like ECH-associated protein 1; ATF4, activating transcription factor 4 (tax-responsive enhancer element B67); JUN, jun oncogene; NFE2, nuclear factor, erythroid 2; MAFG, v-maf musculoaponeurotic fibrosarcoma oncogene homolog G; MAFF, v-maf musculoaponeurotic fibrosarcoma oncogene homolog F; MAPK, v-maf musculoaponeurotic fibrosarcoma oncogene homolog K; CREBBP, CREB binding protein; JUND, jun D proto-oncogene; PRKCD, protein kinase C- δ .

Table 1
Enzymatic and non-enzymatic systems that regulate vascular cell redox balance

Vascular cell redox status is maintained by a series of enzymatic and non-enzymatic antioxidant systems that counterbalance oxidant-generating systems. These functions, mechanisms of action, and contributions of these systems to vascular disease are reviewed in references [6–13].

OXIDANT-GENERATING SYSTEMS
NADPH oxidases
Xanthine oxidase
Lipoxygenase
Cyclooxygenase
Uncoupled eNOS
Mitochondria
ANTIOXIDANTS
<i>Enzymatic antioxidants</i>
Superoxide dismutases
Catalase
Glutathione peroxidases
Glutathione-S-transferases
Thioredoxin
Peroxiredoxins
Glucose-6-phosphate dehydrogenase
<i>Non-enzymatic antioxidants</i>
Lipid soluble
α -tocopherol
β -carotene
lycopene
Water soluble
Glutathione
Ascorbic acid