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Redox sensing: Orthogonal control in cell cycle and apoptosis signaling

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Abstract

Living systems have three major types of cell signaling systems that are dependent upon high-energy chemicals, redox environment and transmembrane ion gating mechanisms. Development of integrated systems biology descriptions of cell signaling require conceptual models incorporating all three. Recent advances in redox biology show that thiol/disulfide redox systems are regulated under dynamic, non-equilibrium conditions, progressively oxidized with the life cycle of cells and distinct in terms of redox potentials among subcellular compartments. The present article uses these observations as a basis to distinguish “redox-sensing” mechanisms, which are more global biologic redox control mechanisms, from “redox signaling”, which involves conveyance of discrete activating or inactivating signals. Both redox sensing and redox signaling use sulfur switches, especially cysteine (Cys) residues in proteins which are sensitive to reversible oxidation, nitrosylation, glutathionylation, acylation, sulfhydration or metal binding. Unlike specific signaling mechanisms, the redox-sensing mechanisms provide means to globally affect the rates and activities of the high-energy, ion gating and redox-signaling systems by controlling sensitivity, distribution, macromolecular interactions and mobility of signaling proteins. Effects mediated through Cys residues not directly involved in signaling means redox-sensing control can be orthogonal to the signaling mechanisms. This provides a capability to integrate signals according to cell cycle and physiologic state without fundamentally altering the signaling mechanisms. Recent findings that thiol/disulfide pools in humans are oxidized with age, environmental exposures and disease risk suggest that redox-sensing thiols could provide a central mechanistic link in disease development and progression.

Keywords

Redox signaling; oxidative stress; glutathione; thioredoxin; apoptosis; cell cycle

Introduction

Proteins are unique among biologic macromolecules in possessing elements (cysteine, methionine, selenocysteine) that undergo reversible oxidation-reduction (redox) reactions as part of their normal function. Early biochemical research elucidated roles for cysteine (Cys) residues of proteins in many catalytic mechanisms, as well as in protein stability through disulfide bond formation and interactions with Zn^{2+} , and defense of cells against oxidants and reactive electrophiles. In recent years, this extensive knowledge of Cys functions in

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proteins has been expanded with the recognition that reversible covalent modifications of specific Cys residues function in signal transduction.

Despite rapid progress in this research, especially in the context of kinase signaling and oxidative mechanisms of disease, there remains a relatively incomplete definition of redox-signaling pathways, i.e., ones that could be termed “canonical” pathways. Certainly, there are some, such as the Nrf-2 transcription factor system controlling antioxidant response genes [1]. In this system, sensory thiols in the actin-associated protein Keap-1 in the cytoplasm release Nrf-2 upon oxidation or modification by alkylating agents. Upon release, Nrf-2 translocates to the nucleus, binds with small Maf proteins and activates transcription through binding to antioxidant response elements (ARE) in the control regions of multiple detoxification genes [1].

Many signaling processes are discussed in terms of “redox signaling” based upon sensitivity to non-specific oxidants and reductants such as H_2O_2 and N-acetylcysteine. However, such redox sensitivity does not constitute a “redox-signaling pathway” in the same sense as that provided by the Nrf2 pathway or as exemplified by multiple kinase signaling pathways. Kinase signaling pathways are often responsive to oxidants and reductants [2], but these redox sensitivities are often difficult to describe in terms of what could be considered a “canonical” redox-signaling pathway.

The purpose of the present article is to review literature on thiol/disulfide systems and the characteristic differences in these which are associated with the life cycle of cells and subcellular compartments. This literature indicates that global redox control mechanisms exist which serve biologic functions different from those provided by specific signal transduction mechanisms. Based upon this, I propose to use the term “**redox sensing**” to describe these more global thiol-dependent control mechanisms and suggest that the term “**redox signaling**” be reserved for mechanisms in which a specific redox element transmits an activating or inactivating signal, i.e., a system for which a canonical redox-signaling pathway can be defined. Although this distinction will be difficult in some instances, I believe that efforts to make this distinction will facilitate development of integrated models of cell signaling which can accommodate the spectrum of functional differences associated with redox compartmentalization and the life cycle of cells.

I start with a brief overview of the Cys proteome and the spectrum of covalent and non-covalent modifications which underlie thiol-dependent mechanisms of signaling and metabolic regulation. The next section summarizes patterns of variation of steady-state redox potentials in the life cycle of cells and related associations with biologic function. This is followed by development of the concept of redox sensing as something distinct from redox signaling, with redox-sensing functions specifically to provide means to coordinate cell signaling and deliver signals appropriate to the cell type, cell cycle and metabolic environment. The final section addresses implications of redox sensing to structure-function relationships in biological systems, highlighting a need for systematic studies of the cysteine proteome to develop integrated systems biology descriptions of cell responses to stress.

The Cysteine Proteome

About 214,000 Cys residues are contained within the proteome encoded by the human genome [3]. Miseta and Csutora [4] studied the relationship between the occurrence of Cys in proteins and the complexity of organisms. They found that when Cys as a percentage of total amino acid content is compared to that expected randomly from the $tRNA^{Cys}/tRNA^{total}$, Cys is underrepresented in the proteome, even when corrected for GC content (0.41% for Archaea to 2.26% for mammals; random probability is 3.28%). They noted in this analysis that 92% of mammalian proteins contain at least 1 Cys, but only 50% of

Archaea proteins have at least 1 Cys, suggesting a change in Cys content with evolution of complexity. This was supported by analysis of 10 ribosomal proteins of 5 species, where they found that Cys content increased with complexity of species (Archaea → *Escherichia coli* → Yeast → *Drosophila* → Human). Cys content for the ribosomal proteins was about half of that for all proteins of the respective species, indicating that there was a selection against Cys content. Neither Phe, Tyr nor Ile had pattern similar to Cys, and Cys appeared in non-random distribution with considerable increase in vicinal dithiol structures, with C-X-X-C content 1.5-times that of C-X-C or C-X-X-X-C. Results showed that 20% of human proteins studied had such a structure. Archaea proteins also had 21% of all Cys in such a structure, but yeast (5%) and plants (<5%) did not have notable occurrence of these structures. The results of these studies indicate that Cys content in animal evolution increased with complexity of the organism.

An increase in Cys content could reflect evolution of signaling and control functions of Cys with evolution of complexity. A large number of functions have been associated with covalent and non-covalent modifications of Cys (Fig 1). Reversible redox reactions can occur with single Cys or with vicinal Cys. Single Cys forms disulfides with GSH, Cys or other proteins while vicinal Cys form disulfides or metal clusters. Sulfenic acids are also formed but are typically unstable and converted to disulfides. Higher oxidation states, i.e., sulfinic and sulfonic acids, largely represent irreversible oxidations in mammalian systems, although reversible oxidation of sulfinic acid in peroxiredoxin can potentially function in redox signaling. Cys undergoes other reactions, such as nitrosylation by NO and sulfhydration by H₂S. Reversible formation of thiolhemiacetals occurs with aldehydes, including monosaccharides, and acylation occurs by reactions with acyl-CoA's. These Cys modifications have a range of functions, including control of protein activity, stabilization of protein structure, formation of protein activation complexes, control of protein distribution in association with the cytoskeleton or membranes, and translocation of proteins through the secretory pathway, into mitochondria or into and out of cell nuclei.

Comparison of the percentage of Cys in the ribosomal proteome as a function of animal evolution, as shown by Miseta and Csutora [4], with the percentage preserved with evolution, shows similar characteristics for both the cytoplasmic and mitochondrial ribosomes (Fig 2A–D), even though the compartments and the functional demands for the respective ribosomes are different. The percentage is substantially less than that expected from random use of the codon, indicating selection against use of Cys. However, most of the Cys present are increasingly preserved among the more complex organisms, suggesting evolution of functions. These characteristics are very different from the mitochondrial targeting sequences (MTS) for the mitochondrial ribosomal subunits (Fig 2E,F), which have a percentage of Cys similar to that expected from random use of the codon and show little evidence for conserved Cys residues. One can conclude that there is selection against random use of Cys in animal protein structures. In other words, Cys residues are used sparingly but are retained within structures during evolution of complexity, a characteristic of functionally important amino acid residues.

Redox proteomics methods allow identification of proteins that undergo redox changes under relevant biologic conditions. Targeted analyses using redox western blotting show that selected proteins in the mitochondria, cytoplasm, nuclei, endoplasmic reticulum and plasma membrane are partially oxidized under normal physiologic conditions [5]. H₂O₂ production rates in cells are sufficient to oxidize about 0.5% of all cell thiols each minute [3], and computational modeling indicates that protein oxidation contributes in a quantitatively important way to elimination of low levels of H₂O₂ [6]. This interpretation is supported by powerful new mass spectrometry (2-D LC-MS/MS) based methods [5], which allow detection of over 2000 specific Cys residues in peptide sequences (Y-M Go & DP Jones,

unpublished). The results show that hundreds of specific Cys residues are partially oxidized and responsive to physiologic state of cells. Importantly, because the methods preferentially detect abundant proteins and capture only about 1% of the proteome, the number of redox-sensitive Cys residues is likely to be much higher.

The specific Cys residues that are observed to be oxidized under normal physiologic conditions are present in a broad spectrum of structural and functional classes. For instance, 34 actin-associated cytoskeletal proteins were found to change in fractional oxidation in aortic endothelial cells exposed to a relatively oxidized extracellular cysteine/cystine redox potential [5]. Other partially oxidized cytoskeletal proteins include tubulins, cofilin and dyneins; signal transduction proteins include Ras, phosphatases, 14-3-3; translation proteins include elongation initiation factors and several ribosomal subunits; cell stress proteins include several heat shock proteins; detoxification proteins include peroxiredoxins and glutathione S-transferases; mitochondrial proteins include ATP synthase subunits and Complex I subunits; metabolic enzymes include several enzymes of fatty acid metabolism; pro-inflammatory signaling proteins include several stimulated by IL-1 β . The major conclusion from these observations is that there is a large and diverse group of proteins with Cys residues that are partially oxidized under physiologic conditions.

Patterns of variation in steady-state redox potentials in cells

Variation with the life cycle of cells

Among the metabolic activities that vary with cell cycle, changes in thiol content have received considerable attention, especially related to efforts to enhance killing in resistant cancer cells. An overview of some of these characteristics is given in Fig 3. Thioredoxins are typically in a more reduced state in both proliferating and differentiated cells compared to apoptotic cells. Depletion of metabolic energy sources causes oxidation. Extensive oxidation caused by prolonged loss of energy precursors, oxidants, certain metal ions and electrophiles, activates cell death through apoptotic and necrotic mechanisms.

Highly proliferative cells have higher GSH concentrations as well as higher thioredoxin concentrations [7–10]. Numerous studies show that depletion of GSH or inhibition of thioredoxin impairs proliferation [7–10]. For conditions where GSH is depleted, restoration of GSH supports recovery of proliferation. Similar studies in normal cells show the same characteristics, and *in vivo* studies in intestinal epithelia show that crypt cells have higher GSH concentrations than non-dividing tip cells [11]. Such studies also show that conditions limiting GSH availability *in vivo* also limit crypt cell division, that proliferation is restored upon restoring GSH supply and that proliferation can be enhanced by supplementation with GSH precursors [12–14]. Changes in GSH within the nuclear compartment in association with proliferation have been recently shown using different GSH-depleting agents; importantly, these data show that maintenance of nuclear GSH is critical for proliferation [15].

While studies of GSH concentration per se show GSH-dependent stimulation of proliferation, measures of redox potential for the GSH/GSSG couple (designated $E_h\text{GSSG}$ for the reduction potential of $\text{GSSG} + 2e^- + 2\text{H}^+ \leftrightarrow 2\text{GSH}$) further support the conclusion that proliferative cells maintain a relatively reduced redox potential (see Fig 3B). In colon carcinoma HT-29 cells, the steady-state $E_h\text{GSSG}$ was -260 mV in rapidly proliferating cells and this shifted to -200 mV in cells treated with butyrate to induce differentiation [16]. Colon carcinoma Caco2 cells similarly showed an $E_h\text{GSSG}$ of -245 mV which changed to -205 mV following reaching confluence and undergoing spontaneous differentiation [17]. *In vivo* studies in rat colon have shown similar changes in redox potential associated with

proliferation and growth arrest [18]. NIH3T3 fibroblasts show similar differences between proliferating and differentiated cells [19].

Less information is available concerning the relationship between cellular Cys and proliferation. Cys is required for protein synthesis, and cell proliferation requires protein synthesis. Because of the metabolic interconversion of GSH and Cys, there is a possibility that Cys could be limiting under some conditions previously associated with GSH limitation. Measures of E_h CySS show an oxidation with differentiation, although the magnitude of change is not as great as for E_h GSSG [20]. On the other hand, extracellular E_h CySS has been found to be a critical determinant of proliferation in many cell types [21]. For instance, a more reduced extracellular E_h CySS has been found to stimulate proliferation in monocytes [22], endothelial cells [23], retinal pigment epithelial cells [24] and multiple colon cancer cell lines [25]. Cys and CySS constitute the major low molecular weight thiol/disulfide system in many extracellular fluids, so that one can conclude that Cys/CySS is a critical extracellular thiol/disulfide couple controlling cell proliferation and provides a complement to the cellular GSH/GSSG system in this regulatory function.

Oxidation of thiol systems has long been known to cause cell death due to dysregulation of Ca^{2+} homeostasis, activation of the mitochondrial permeability transition and activation of apoptosis signal-regulating kinase-1. During apoptosis, GSH efflux is activated [26] so that regardless of how apoptosis is signaled, a common feature is oxidation to about -170 mV. For instance, oxidation of E_h occurs during apoptosis induced by staurosporin [27], glucocorticoid [28], nutrition factor withdrawal [29], or terminal differentiation [17]. Oxidation of E_h GSSG to values more positive than -150 mV cause necrosis, and secondary necrosis is often observed in cell culture where apoptotic cells are not removed by phagocytosis.

Oxidized extracellular E_h CySS activates proinflammatory signaling in endothelial cells, monocytes and neutrophils [23,30]. Stress response and antioxidant response pathways are also activated by oxidized E_h CySS in monocytes [22], endothelial cells [23], colonic epithelial cells [31], and profibrotic signaling is activated in pulmonary fibroblasts [32]. Although direct mechanistic links of these changes with disease pathology are limited, studies in humans show associations of oxidized E_h CySS with risk of persistent atrial fibrillation [33] and cardiovascular disease outcome [34].

Additional studies are needed to determine characteristics of other tissues and biologic conditions. However, at the current level of knowledge, data suggest that the redox potential values are sufficiently consistent to consider the possibility that thiol/disulfide redox potential is an underlying parameter contributing to the organization and control of molecular activities during the life cycle of cells.

Subcellular compartmentation of redox

Available information concerning redox compartmentalization has been recently reviewed [21] and also supports the interpretation that the redox potentials of thiol/disulfide systems provide a basis for organization and control of molecular activities. Early studies on GSH showed only small differences between mitochondria and cytoplasm, but these studies did not include the consideration of a pH difference between the compartments, which makes the E_h GSSG more reducing in the mitochondria even with the same GSH/GSSG ratio. Early findings of high GSH in nuclei [35] were discounted because of possible artifacts related to method of analysis [36]. However, studies of transcriptional activation by AP1 showed that low level of oxidation stimulated activation while high level of oxidation inhibited activation [37–39], and these results were shown to be due to different oxidative events in the cytoplasm and nuclei. Similar redox sensitivities are known for NF- κ B and Nrf-2 [40–

42]. Subsequent studies have confirmed that the GSH pool in nuclei is distinct from the cytoplasm [15]. Among the most convincing early evidence for redox compartmentalization, thioredoxin-1 was found to be translocated into the nucleus in response to oxidative stress [43]. Early studies also showed redox compartmentalization in the endoplasmic reticulum. E_h values were obtained with an ER-targeted peptide and showed that the E_h GSSG in the cisternae is relatively oxidized [44], consistent with the substantial knowledge of the oxidative protein processing in proteins destined for secretion [45].

A diagram illustrating the compartmentalization of thiol/disulfide redox potentials is given in Fig 4. This is presented conceptually because different thiol-disulfide couples were measured in different compartments; a recent review of this subject is available [21] and should be consulted for details. Calculation of steady-state redox potentials of Trx1 and Trx2 using measured fractional reduction and appropriate standard potentials for the active site dithiol/disulfide couples, show that the mitochondrial Trx2 is maintained at the most reducing potential (-330 mV), with nuclear Trx1 being about -300 mV and cytoplasmic Trx1 about -270 mV. The E_h GSSG in the mitochondria is the most reducing of the GSH/GSSG pools, being about -300 mV. The E_h GSSG in the nucleus is not known, but evidence that GSH concentration is higher than in the cytoplasm and that the ratio of protein thiol to glutathionylated protein ($-SH/\text{protein-SSG}$ ratio) is higher for nuclear proteins than for cytoplasmic proteins, suggest that the E_h GSSG is more negative than the cytoplasmic values, i.e., <-260 mV. Values for the cisternae of the endoplasmic reticulum obtained by Hwang et al [44] are approximately -185 mV, intermediate between cytoplasmic (-260 to -200 mV) and plasma values (-140 mV).

Several correlations between compartmental redox potential changes and cell function are known (summarized in [21]). Cytoplasmic activation of signaling by AP1, NF- κ B and Nrf2 pathways and sensitivity of these pathways to oxidative inactivation of transcription in nuclei have been well documented [39,41,43]. Growth signaling by EGF has been found to involve selective oxidation of the cytoplasmic Trx1 pool without oxidation of cellular GSH, nuclear Trx1 or mitochondrial Trx2 [46]. Signaling by TNF- α was associated with oxidation of mitochondrial Trx2 without oxidation of cytoplasmic Trx1 or cellular GSH/GSSG [47]. Similarly, proinflammatory signaling by oxidized extracellular E_h CySS in endothelial cells was associated with mitochondrial Trx2 oxidation but not oxidation of cytoplasmic Trx1, nuclear Trx1 or cellular GSH/GSSG [5]. The selective oxidation within compartments suggests that redox potentials are controlled independently in a manner which could allow optimization of protein functions. Together with the above data showing thiol/disulfide redox potential changes with the life cycle and a spectrum of reversible modifications of Cys in proteins, the data suggest that redox elements provide means to both support biologic signaling and also to coordinate signaling functions in the cell cycle and apoptosis.

Discrimination of redox sensing and redox-signaling thiols

In an earlier review of the evidence for non-equilibrium thermodynamics of thiol/disulfide systems, we provided a perspective on redox systems biology and the need to develop quantitative models that accurately describe function of redox signaling and control pathways [18]. One of the major challenges identified was to discriminate between sulfur switches that function as integral components of redox-signaling pathways and those that function to control activity of signaling pathways. This challenge is exemplified by the need indicated above to identify canonical redox-signaling pathways, few of which have been clearly defined.

“Redox signaling” is often used to describe signaling processes in which an oxidant such as H_2O_2 generates a biologic response. Nrf2 is a prototypic redox-signaling pathway [1,48].

Nrf2 is bound to the actin-associated binding protein Keap-1 in the cytoplasm. Oxidation or alkylation of Cys residues in Keap-1 provides a signal to release Nrf2, which translocates to the nucleus and activates transcription via antioxidant response elements (ARE) within the control regions for antioxidant and detoxification genes. A large number of other redox-sensitive transcription factors have been identified [49], but few of these have well defined, discrete redox-signaling pathways in which a specific oxidative signal is conveyed through a specific redox element to direct a specific cellular response.

In contrast, there are multiple examples where kinase pathways are known to be redox sensitive. For instance, Wright et al [2] found that low level oxidative stress increased phosphorylation of 85% of the phosphorylated proteins that they were able to detect. Many studies show that oxidative conditions often stimulate kinase reactions while at the same time inhibiting phosphatase reactions [50,51]. The implication from the relatively few discrete redox-signaling pathways and the common cross-talk between redox and kinase mechanisms is that there is a need to distinguish elements of discrete signaling pathways from elements which regulate those pathways. This need is especially relevant to processes designated as “redox signaling” based upon experiments with H₂O₂, N-acetylcysteine or other non-specific oxidant or reductant. For instance, experiments with additions of 200 μM or more H₂O₂ or 1 mM or more NAC, to cell incubations where total thiol content is in the range of 100 μM or lower, provides no evidence for a specific signaling pathway. On the other hand, the responses observed with such global oxidative challenges may be important evidence for control mechanism exerted through **redox-sensing** thiols rather than **redox-signaling** thiols.

If one takes a very broad view, cell signaling and metabolic regulatory mechanisms can be linked to the three inter-convertible energy currencies, i.e., phosphorylation energy, transmembranal gradient energy and redox energy (Fig 5). Each energy currency underlies types of cell signaling mechanisms represented by kinase signaling, transmembranal ion signaling and redox signaling. However, because the signaling pathways are regulated, and the underlying mechanisms of regulation of these pathways are based upon the same energy currencies, the discrimination of discrete signaling pathways from the systems that regulate those pathways is difficult.

In the context of the data summarized above, namely that 1) there are many Cys residues in proteins, 2) a considerable fraction of these Cys residues undergo reversible oxidation or other covalent modification, 3) redox potentials within cells undergo characteristic changes associated with the life cycle of cells and 4) subcellular compartments are specialized in their redox control systems, one can conclude that categorizing protein Cys residues according to their function could be very useful. To accommodate the effects of global redox changes, such as those observed during cell differentiation and apoptosis, and the concepts of cell signaling and signal transduction, some clarification of terminology is needed. One way to do this is to use the terminology for Cys residues functioning as essential elements in redox-signaling pathways as “**redox-signaling**” Cys while Cys residues functioning to regulate or integrate signaling functions, regardless of signaling pathway, could be considered “**redox-sensing**” Cys. This distinction is depicted schematically in Fig 6, where discrete signaling pathways are designated by the arrows directed toward the lower right and redox regulatory systems for these pathways are designated by arrows to the lower left. In this depiction, which is intended to provide a conceptual basis for discriminating redox-sensing Cys from redox-signaling Cys, I have also included kinase and transmembranal ion signaling pathways to emphasize that there are redox-sensing mechanisms regulating each signaling type. For instance, in Trx1-dependent signaling of apoptosis by Ask-1, oxidation of Cys32,35 results in signaling of the pathway [52] while oxidation of redox-sensing Cys62,69 serves to regulate that activation by controlling the rate of reduction of the

Cys32,35 disulfide [53]. Kinase activation as described above is regulated by multiple redox-sensing mechanisms [2,54], and ion movement through the NMDA (N-methyl-D-aspartate) receptor is similarly regulated by redox-sensing thiols on the extracellular face of the ion channel [55].

Redox-sensing thiols can alter the rates or conditions in which a signaling pathway can function without fundamentally altering the signaling mechanism. Consequently, the function of the redox-sensing thiols can be viewed as being orthogonal to the signaling pathway (Fig 6). This allows redox-sensing thiols to control signaling in a manner appropriate to the cell conditions so long as the magnitude of redox potential change is sufficient. Because the E_h is related to the logarithm of disulfide/thiol for single Cys and to the logarithm of disulfide/(thiol)² for Cys pairs, a 60 mV change is equivalent to a 10-fold change in reduced:oxidized ratio for single Cys and a 100-fold change in ratio for dithiol motifs. In HT-29, a 60 mV oxidation was observed in association with differentiation [16]. This change considerably enhanced the sensitivity of the Nrf-2 system and thereby controlled the extent of transcriptional activation signaled in response to benzyliothiocyanate. This resulted in higher expression of detoxification genes in terminally differentiated cells compared to rapidly dividing cells [16]. As summarized in Fig 3, the magnitudes of change during the cell cycle, differentiation, and apoptosis are sufficient for redox-sensing thiols to have considerable effect on cellular structure and activities.

Thus, the shift in redox potential, as is commonly observed during differentiation or apoptosis, provides a mechanism to control signaling pathways appropriately for dividing, differentiated and apoptotic cells. More reduced conditions can enhance cell proliferation in cancer cells by simultaneously affecting the activity of multiple cell survival genes. Such distinction between redox-signaling pathways and redox-sensing mechanisms not only will help to accurately describe pathways but will also facilitate development of accurate mathematical descriptions in redox systems biology.

Implications of redox-sensing Cys in biological systems

The observation that the percentage of Cys in proteins is less than that expected from random use of the Cys codon suggests that the properties of Cys have had a considerable influence on the evolution of the Cys proteome. The multiple reactions of the Cys thiols and the sensitivity of thiols to oxidation and reaction with reactive electrophiles serve to reinforce this concept. Furthermore, extensive toxicology and carcinogenesis literature shows the direct importance of the Cys proteome in biologic function. The amount of oxidant needed to disrupt a specific signaling process, however, should be small relative to the amount needed to affect the global Cys proteome of cells, and the amounts of H₂O₂ or NAC that have been used to define “redox signaling” are sufficient to affect the global proteome. The biological literature has largely ignored this inconsistency and/or relegated the global oxidation of proteins to a role in tolerance to oxidative challenge, i.e., an evolved strategy to protect critical active site Cys from oxidation by having a large number of non-critical Cys and Met residues which preferentially react with oxidants [56].

An alternate interpretation based upon the distinction between redox-signaling and redox-sensing Cys and the observed characteristics of the Cys proteome is that a large fraction of the Cys present in mammalian systems has redox-sensing function. Many examples can be given to illustrate this point. For instance, disruption of disulfide formation in protein processing through the secretory pathway, activates the ER stress response [57,58]. In intestinal and pulmonary mucus, oxidation of Cys results in a more viscous and rigid barrier against microorganisms [59]. In platelet activation, a balance of GSH and GSSG is needed

for optimum signaling [60]. An optimum redox potential in cell culture medium is required to achieve maximum number of cells (cell density) in mammalian cell culture [25]. A high concentration of GSH is required for cell proliferation [15], and a more reduced E_hCySS signals cell proliferation [61].

Such examples of redox-sensing functions can occur through known characteristics of thiols. Cytoskeletal proteins contain conserved Cys residues, some of which have redox-dependent functions [62]. During oxidative challenge, actin is extensively oxidized, crosslinked and bound to many proteins [63,64]. At the physiologic limit, oxidation, crosslinking and protein binding could serve to control function, distribution and interaction of associated proteins. In other words, the toxicologic response due to global protein oxidation may reflect physiologic redox-sensing mechanisms which are overwhelmed by global oxidation.

A similar interpretation can be given to α - and β -tubulin, docking proteins such as 14-3-3, chaperones such as HSP60 and HSP90, translation machinery such as EF1 α and EF2, protein degradation components such as ubiquitin-activating E1, and other proteins which are found to be partially oxidized under normal physiologic conditions in cells [5]. Dynamic redox responses of such proteins could reflect redox-sensing functions which serve to coordinate and organize the protein machinery. The known examples of thiols in protein processing and trafficking [45] provide a proof-of-principle for such mechanisms. The presence of a large number of proteins that are partially oxidized under physiologic conditions and the observation that thiol/disulfide systems are maintained under non-equilibrium states almost necessitates that such effects are present, even if they are not used biologically.

Older literature on Ca²⁺ diffusion in cytoplasm [65,66] suggests a possible mechanistic basis for redox-sensing Cys to be used in coordination and organization of cell functions. The apparent diffusion coefficient of Ca²⁺ in cytoplasm is considerably less than in a simple salt solution and also considerably less than that of other cations. This characteristic is due to the sequestration of Ca²⁺ which causes up to a 50-fold decrease in the apparent diffusion coefficient [65,66]. A similar effect on diffusion of specific proteins could occur because thiol-disulfide exchange is a relatively slow process. Under a relatively reducing condition compared to that which results in inter-protein disulfide bond formation, translational movement of proteins would not be affected by protein-protein interactions. However, if conditions were appropriate for reversible inter-protein disulfide bond formation with membranal or cytoskeletal proteins, then the translational movement of a soluble protein would be slowed. Translational movement would be determined by the rate of thiol/disulfide exchange with the immobile thiols.

The potential impact of such a mechanism is illustrated in Fig 7. Thiol/sulfide systems are maintained under non-equilibrium states, but if the sites of oxidation or reduction are homogeneously distributed, the cytoplasmic redox potential will be uniform throughout the compartment (Fig 7A). If local oxidants are produced at regions of the plasma membrane, as suggested to provide specificity in redox signaling [48], then localized oxidation could transiently inactivate phosphatases and reductases within the region, and enhance this effect by transiently slowing diffusion of proteins in the region of the signal generation (Fig 7B). Similarly, if mitochondria are stimulated to produce oxidants, these could generate a zone of oxidation that activates signaling mechanisms in the vicinity of individual mitochondria (Fig 7B). In this way, Cys residues which have been thought to have no biologic function based upon effects from site-directed mutation could none-the-less have a biologic function. Furthermore, when proteins are studied in purified form in solution, such a function would be difficult to detect. Global effects of H₂O₂ or thiol reductants, as well as nitrosylation, sulfhydration and similar processes, could affect signaling pathways by such mechanisms.

Hypothetical roles of such redox-sensing mechanisms in cellular organization are presented in Fig 7C and 7D. Because the nuclei are more reduced and the extracellular space is more oxidized than cytoplasm, the non-equilibrium conditions of the thiol/disulfides could exist with a radial gradient in which the distribution, mobility and/or activity of proteins is sorted according to the prevailing local redox potential (Fig 7C). Such a mechanism could provide a basis for polarity within the cytoplasm. Similarly, transcellular redox potential gradients can exist in transport epithelia [67, 68] and these could provide a basis for sorting of proteins and their activities across the cell (Fig 7D). Although speculative, the presence of a large number of redox-sensing Cys within cellular proteins could explain a considerable oxidative stress literature in which global oxidative challenges impact specific signaling mechanisms.

Challenges and opportunities

Studies of cells transfected with Nox-1 showed activation of kinase signaling without detectable changes in redox states of GSH/GSSG or thioredoxin-1 (Trx1) [69]. Such research implies that redox signaling is specific and occurs without global changes in the major thiol/disulfide systems. Furthermore, the disequilibrium of steady-state redox potentials of cysteine/cystine, GSH/GSSG and protein thiol/disulfide couples show that thiol/disulfide systems interact too slowly to allow generalized thiol/disulfide “buffering”. Advances in mass spectrometry-based redox proteomics shows that hundreds, and perhaps thousands, of specific protein Cys residues are partially oxidized under normal *in vivo* conditions. Thus, central methodologic challenges to understand redox signaling and redox-sensing mechanisms are to discern oxidation of specific protein thiols from among the 214,000 Cys residues of proteins encoded by the mammalian genome. To do this effectively, cumulative databases will be needed in which different subsets are measured in individual experiments and assembled to give a more complete understanding of the redox-sensitive proteome. Ultimately, methods will be needed to resolve such redox events at the sub-micron scale of cells.

Recent progress in development of methods to measure oxidation of specific amino acid residues in specific proteins by mass spectrometry now allow measurement of about 1% of the Cys proteome in a single experiment, and a range of redox western blots are available to measure oxidation of selected proteins [70,71]. Fluorescence methods using redox-sensitive fluorescent proteins allow imaging of redox changes within living cells [72], and nano-technology has provided the promise of being able to measure redox signaling with nano-scale resolution [73]. Thus, challenges that have limited progress in understanding the Cys proteome are being surmounted, portending a rapid maturation of the research subject.

The capability to accurately measure oxidants remains limited, but progress has been made especially for H₂O₂ and hydroperoxides [74]. However, there is a considerable challenge to spatially resolve low concentrations of oxidants that have short half-life. Furthermore, there has been little progress in sorting out relevant oxidants from all of the possible oxidants which could function in redox signaling and redox sensing. Even the most actively studied reactive oxygen species (ROS) are not always distinguished as H₂O₂, lipid hydroperoxide or superoxide anion radical. And other possible oxidants, such as quinones, endoperoxides, epoxides, aldehydes, sulfites, chloramines, disulfides and molecular O₂, are often not considered. With rapidly emerging proteomics capabilities, greater opportunity to study the spectrum of oxidants functioning in redox signaling will be available.

Finally, consideration of redox-sensing thiols as a common component of the Cys proteome provides an opportunity to correct persistent misinterpretations about oxidative reactions in biologic systems. In particular, the functional roles of thiol-dependent redox reactions have been overshadowed by the free radical theory of aging and related hypotheses which

hypothesize and conclude that essentially everything oxidative is bad. Such studies often obscure normal physiology by creating experimental conditions where destructive 1-electron chemistry predominates and/or uncontrolled free radical reactions occur because cells are dead [75].

The fact that proteins become oxidized and lose activity during tissue fractionation [76] has often considered “artifact” without questioning why evolution produced such reactive and unstable proteins. The rates of autooxidation of Cys thiols in proteins are similar to rates expected from oxidative turnover of H_2O_2 in cells [3]. Because the function of redox-sensing thiols is dependent upon being oxidizable, these Cys can be expected to undergo oxidation during extraction and separation from redox-control systems. With this interpretation, inactivation by autooxidation provides a basis to study a protein for the presence of redox-sensing thiols. Examination of this subset of the Cys proteome that readily undergoes reversible oxidation-reduction under physiologic conditions as redox-sensing elements creates tremendous opportunity to enhance the understanding of the signaling and control of the cell cycle and apoptosis.

Clinical implications

Recognition that a relatively large number of redox-sensing thiols exist in biologic systems explains the dissociation between basic and clinical findings that developed with the failure of large-scale, double-blind antioxidant trials to show significant health benefit [79–86]. Oxidative stress is associated with diverse disease processes and, by most criteria, can be interpreted to contribute in a causal way. The results of the rigorously designed trials have led many to question the large volume of data concerning oxidative mechanisms of disease. However, oxidative stress involves free radical and non-radical mechanisms [3]. The antioxidant trials used free radical scavenging antioxidants, yet free radicals represent only a small fraction of the oxidants produced in biologic systems [3]. Furthermore, free radical scavengers convert free radicals to non-radical oxidants. Thus, non-radical oxidants, such as H_2O_2 , are more abundant than free radicals. Non-radical oxidants selectively oxidize thiols rather than causing indiscriminant macromolecular damage. This results in more nuanced pathophysiologic mechanisms than a simplistic interpretation that more oxidation is “bad” and less is “good”.

Accumulated scientific knowledge shows that thiol/disulfide pools in humans are oxidized with age, conditions of oxidative stress (e.g., smoking, alcohol abuse) and other common determinants of chronic disease (e.g., high BMI, endothelial dysfunction) [3]. Oxidized extracellular $\text{E}_\text{h}\text{CySS}$ is sufficient to activate proinflammatory cytokine production in cell culture, and proinflammatory cytokine levels in humans are increased in association with oxidized $\text{E}_\text{h}\text{CySS}$ [30]. Thus, oxidized thiol/disulfide couples, acting through redox-sensing thiols, provides a mechanistic link between the abundant causes of oxidative stress and the underlying cell signaling and regulatory mechanisms that determine disease progression.

We do not presently know what controls the redox balance of the thiol/disulfide systems in humans. Recent evidence is available that dietary sulfur amino acid intake can affect plasma redox status in the short term [88,89] and that Zn^{2+} supplementation and a combination of vitamin C and E can protect against an oxidation over a 5-year period in older individuals [90,91]. It would appear that systemic oxidation of thiol/disulfide couples could impact many systems because of the presence of redox-sensing thiols. In this way, cell proliferation and repair mechanisms may decline with age and oxidative stress conditions. Cells would respond to oxidized E_h through redox-sensing thiols with increased expression of pro-apoptotic machinery, shifting the balance of cell populations. Processes such as platelet

activation, fibrotic signaling and inflammatory signaling would be sensitized by redox-sensing thiols in the signal transduction pathways.

Knowledge of redox-sensing thiols will support new strategies to address disease risk and the development and management of disease. Application of new technologies to define redox-sensing thiols will provide the basis for development of new families of antioxidants targeted to relevant redox systems and disease pathways. Although the potential benefits are largely conjectural at present, the progress in understanding thiol/disulfide changes in vascular disease [Go and Jones, manuscript submitted], where outcome measures are directly linked to oxidized thiol/disulfide systems, raises the hope that the new insight concerning redox-sensing thiols will ultimately lead to significant health benefits.

Conclusion

The rapid progress in understanding oxidant generation by NADPH oxidases, redox signaling by Nrf2, and the elucidation of interactions of redox signaling with kinase signaling, has elevated redox signaling to a level of scientific maturity. The present article reviews data concerning the characteristics of the Cys proteome which suggests a need to discriminate more global redox-sensing mechanisms from those involved in specific redox-signaling pathways. The key observations are: 1) characteristic redox potentials occur in proliferating, differentiated and apoptotic cells and also in specific subcellular compartments, 2) thiol/disulfide couples are maintained under non-equilibrium conditions in cells and 3) a large fraction of thiols readily undergo reversible oxidation under physiologic conditions. Consequently, an underlying organizing principle to coordinate multiple biologic processes is likely to involve redox-sensing mechanisms dependent upon thiols. By operating through distinct Cys residues, such control can be orthogonal to cell signaling mechanisms, allowing control of signaling without altering the mechanisms of signaling.

An implication of the interpretation that the Cys proteome contains a subset of redox-sensing Cys that are distinct from the redox-signaling Cys, is that the redox-sensing functions could provide an organizational principle for cells. Although delineation of this subset of thiols is incomplete, mass spectrometry-based redox proteomics suggest that 1% or more of the Cys residues in proteins are partially oxidized and could serve redox-sensing functions. Such redox-sensing thiols could optimize structure-function relationships and account for a large number of reversible oxidation, nitrosylation, acylation, sulhydration or metal binding reactions affecting macromolecular structure, activity, interactions and trafficking.

Localized generation of oxidants and specificity for reduction by thioredoxins, glutathione and Cys couples supports both global and local control structures, thereby affording specificity to high-energy chemical and ion-gating mechanisms, as well as redox signaling. Redox changes during the life cycle of cells can provide conditional control of signaling activities, thereby limiting certain activities to proliferating cells while others are limited to differentiated or apoptotic cells. Compartmentalization of the redox state can provide further specificity to kinase and ion-gating signaling during life cycle transitions. Progress in delineating the redox-sensitive Cys proteome, the associated redox circuits controlling these elements and the spatial and temporal variations in redox state, can be expected to allow incorporation of redox-sensing mechanisms with high-energy chemical and ion gating mechanisms to create integrated systems biological models of cell signaling and control.

Finally, accumulating data shows that oxidation of thiol/disulfide couples in human plasma is a hallmark of aging and a common feature of age-related disease. Relatively little is known about the determinants of steady-state redox potentials in humans, but available data

show a dependence upon diet. Consequently, one can anticipate that management strategies can be developed to protect against oxidation of thiol/disulfide couples and associated redox-sensing thiols. Such approaches can then be applied to test efficacy in disease processes associated with oxidative stress and inflammation.

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References

1. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol.* 2007; 47:89–116. [PubMed: 16968214]
2. Wright VP, Reiser PJ, Clanton TL. Redox modulation of global phosphatase activity and protein phosphorylation in intact skeletal muscle. *J Physiol.* 2009; 587:5767–81. [PubMed: 19841000]
3. Jones DP. Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol.* 2008; 295:C849–68. [PubMed: 18684987]
4. Miseta A, Csutora P. Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol Biol Evol.* 2000; 17:1232–9. [PubMed: 10908643]
5. Go YM, Park H, Koval M, et al. A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential. *Free Radic Biol Med.* 2009
6. Adimora NJ, Jones DP, Kemp ML. A model of redox kinetics implicates the thiol proteome in cellular hydrogen peroxide responses. *Antioxid Redox Signal.*
7. Chang WK, Yang KD, Shaio MF. Lymphocyte proliferation modulated by glutamine: involved in the endogenous redox reaction. *Clin Exp Immunol.* 1999; 117:482–8. [PubMed: 10469051]
8. Kang YJ, Feng Y, Hatcher EL. Glutathione stimulates A549 cell proliferation in glutamine-deficient culture: the effect of glutamate supplementation. *J Cell Physiol.* 1994; 161:589–96. [PubMed: 7962140]
9. Otto WR, Rao J, Cox HM, et al. Effects of pancreatic spasmolytic Polypeptide (PSP) on epithelial cell function. *Eur J Biochem.* 1996; 235:64–72. [PubMed: 8631368]
10. Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol.* 2001; 41:261–95. [PubMed: 11264458]
11. Cornell JS, Meister A. Glutathione and gamma-glutamyl cycle enzymes in crypt and villus tip cells of rat jejunal mucosa. *Proc Natl Acad Sci U S A.* 1976; 73:420–2. [PubMed: 1755]
12. Jonas CR, Estivariz CF, Jones DP, et al. Keratinocyte growth factor enhances glutathione redox state in rat intestinal mucosa during nutritional repletion. *J Nutr.* 1999; 129:1278–84. [PubMed: 10395587]
13. Nkabyo YS, Gu LH, Jones DP, Ziegler TR. Thiol/Disulfide redox status is oxidized in plasma and small intestinal and colonic mucosa of rats with inadequate sulfur amino Acid intake. *J Nutr.* 2006; 136:1242–8. [PubMed: 16614411]
14. Tian J, Washizawa N, Gu LH, et al. Stimulation of colonic mucosal growth associated with oxidized redox status in rats. *Am J Physiol Regul Integr Comp Physiol.* 2007; 292:R1081–91. [PubMed: 17095654]
15. Markovic J, Mora NJ, Broseta AM, Gimeno A, de-la-Concepcion N, Vina J, Pallardo FV. The depletion of nuclear glutathione impairs cell proliferation in 3T3 fibroblasts. *PLoS One.* 2009; 4:e6413. [PubMed: 19641610]
16. Kirlin WG, Cai J, Thompson SA, Diaz D, Kavanagh TJ, Jones DP. Glutathione redox potential in response to differentiation and enzyme inducers. *Free Radic Biol Med.* 1999; 27:1208–18. [PubMed: 10641713]
17. Nkabyo YS, Ziegler TR, Gu LH, Watson WH, Jones DP. Glutathione and thioredoxin redox during differentiation in human colon epithelial (Caco-2) cells. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283:G1352–9. [PubMed: 12433666]

18. Kemp M, Go YM, Jones DP. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med.* 2008; 44:921–37. [PubMed: 18155672]
19. Hutter DE, Till BG, Greene JJ. Redox state changes in density-dependent regulation of proliferation. *Exp Cell Res.* 1997; 232:435–8. [PubMed: 9168823]
20. Jones DP, Go YM, Anderson CL, Ziegler TR, Kinkade JM Jr, Kirlin WG. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *Faseb J.* 2004; 18:1246–8. [PubMed: 15180957]
21. Go YM, Jones DP. Redox compartmentalization in eukaryotic cells. *Biochim Biophys Acta.* 2008
22. Go YM, Craige SE, Orr M, Gernert KM, Jones DP. Gene and protein responses of human monocytes to extracellular cysteine redox potential. *Toxicol Sci.* 2009; 112:354–62. [PubMed: 19748993]
23. Go YM, Jones DP. Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation.* 2005; 111:2973–80. [PubMed: 15927968]
24. Jiang S, Moriarty-Craige SE, Orr M, Cai J, Sternberg P Jr, Jones DP. Oxidant-induced apoptosis in human retinal pigment epithelial cells: dependence on extracellular redox state. *Invest Ophthalmol Vis Sci.* 2005; 46:1054–61. [PubMed: 15728565]
25. Hwang, C.; Sinskey, AJ. Production of biologicals from animal cells in culture. Spier, RE.; Griffiths, JB.; Meignier, B., editors. Oxford: Halley Court; 1991. p. 548-67.
26. van den Dobbelsteen DJ, Nobel CS, Schlegel J, Cotgreave IA, Orrenius S, Slater AF. Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *J Biol Chem.* 1996; 271:15420–7. [PubMed: 8662848]
27. Cai J, Jones DP. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J Biol Chem.* 1998; 273:11401–4. [PubMed: 9565547]
28. Jones DP, Maellaro E, Jiang S, Slater AF, Orrenius S. Effects of N-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol Lett.* 1995; 45:205–9. [PubMed: 7558175]
29. Armstrong JS, Whiteman M, Yang H, Jones DP, Sternberg P Jr. Cysteine starvation activates the redox-dependent mitochondrial permeability transition in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2004; 45:4183–9. [PubMed: 15505073]
30. Iyer SS, Accardi CJ, Ziegler TR, et al. Cysteine redox potential determines pro-inflammatory IL-1 β levels. *PLoS One.* 2009; 4:e5017. [PubMed: 19325908]
31. Nkabyo YS, Go YM, Ziegler TR, Jones DP. Extracellular cysteine/cystine redox regulates the p44/p42 MAPK pathway by metalloproteinase-dependent epidermal growth factor receptor signaling. *Am J Physiol Gastrointest Liver Physiol.* 2005; 289:G70–8. [PubMed: 15746213]
32. Ramirez A, Ramadan B, Ritzenthaler JD, Rivera HN, Jones DP, Roman J. Extracellular cysteine/cystine redox potential controls lung fibroblast proliferation and matrix expression through upregulation of transforming growth factor-beta. *Am J Physiol Lung Cell Mol Physiol.* 2007; 293:L972–81. [PubMed: 17644756]
33. Neuman RB, Bloom HL, Shukrullah I, Darrow LA, Kleinbaum D, Jones DP, Dudley SC Jr. Oxidative stress markers are associated with persistent atrial fibrillation. *Clin Chem.* 2007; 53:1652–7. [PubMed: 17599958]
34. Patel RS, Veledar E, Patel RB, et al. The Oxidized Disulphide Cystine Predicts Adverse Long Term Cardiovascular Outcomes. *Circulation.* 2009; 120:S454.
35. Bellomo G, Vairetti M, Stivala L, Mirabelli F, Richelmi P, Orrenius S. Demonstration of nuclear compartmentalization of glutathione in hepatocytes. *Proc Natl Acad Sci U S A.* 1992; 89:4412–6. [PubMed: 1584774]
36. Briviba K, Fraser G, Sies H, Ketterer B. Distribution of the monochlorobimane-glutathione conjugate between nucleus and cytosol in isolated hepatocytes. *Biochem J.* 1993; 294 (Pt 3):631–3. [PubMed: 8379916]
37. Abate C, Curran T. Encounters with Fos and Jun on the road to AP-1. *Semin Cancer Biol.* 1990; 1:19–26. [PubMed: 2133107]

38. Abate C, Luk D, Gagne E, Roeder RG, Curran T. Fos and jun cooperate in transcriptional regulation via heterologous activation domains. *Mol Cell Biol.* 1990; 10:5532–5. [PubMed: 2119000]
39. Abate C, Patel L, Rauscher FJ 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. *Science.* 1990; 249:1157–61. [PubMed: 2118682]
40. Galter D, Mihm S, Droge W. Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. *Eur J Biochem.* 1994; 221:639–48. [PubMed: 8174544]
41. Hansen JM, Watson WH, Jones DP. Compartmentation of Nrf-2 redox control: regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. *Toxicol Sci.* 2004; 82:308–17. [PubMed: 15282410]
42. Matthews JR, Kaszubska W, Turcatti G, Wells TN, Hay RT. Role of cysteine62 in DNA recognition by the P50 subunit of NF-kappa B. *Nucleic Acids Res.* 1993; 21:1727–34. [PubMed: 8493089]
43. Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem.* 1999; 274:27891–7. [PubMed: 10488136]
44. Hwang C, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science.* 1992; 257:1496–502. [PubMed: 1523409]
45. Cenci S, Sitia R. Managing and exploiting stress in the antibody factory. *FEBS Lett.* 2007; 581:3652–7. [PubMed: 17475256]
46. Halvey PJ, Watson WH, Hansen JM, Go YM, Samali A, Jones DP. Compartmental oxidation of thiol-disulphide redox couples during epidermal growth factor signalling. *Biochem J.* 2005; 386:215–9. [PubMed: 15647005]
47. Hansen JM, Zhang H, Jones DP. Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor-alpha-induced reactive oxygen species generation, NF-kappaB activation, and apoptosis. *Toxicol Sci.* 2006; 91:643–50. [PubMed: 16574777]
48. D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol.* 2007; 8:813–24. [PubMed: 17848967]
49. Allen RG, Tresini M. Oxidative stress and gene regulation. *Free Radic Biol Med.* 2000; 28:463–99. [PubMed: 10699758]
50. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279:L1005–28. [PubMed: 11076791]
51. Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell.* 2005; 121:667–70. [PubMed: 15935753]
52. Saitoh M, Nishitoh H, Fujii M, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J.* 1998; 17:2596–606. [PubMed: 9564042]
53. Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, Jones DP. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. *J Biol Chem.* 2003; 278:33408–15. [PubMed: 12816947]
54. Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol.* 2004; 287:C246–56. [PubMed: 15238356]
55. Choi YB, Lipton SA. Redox modulation of the NMDA receptor. *Cell Mol Life Sci.* 2000; 57:1535–41. [PubMed: 11092448]
56. Bender A, Hajieva P, Moosmann B. Adaptive antioxidant methionine accumulation in respiratory chain complexes explains the use of a deviant genetic code in mitochondria. *Proc Natl Acad Sci U S A.* 2008; 105:16496–501. [PubMed: 18946048]
57. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal.* 2007; 9:2277–93. [PubMed: 17979528]
58. Todd DJ, Lee AH, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol.* 2008; 8:663–74. [PubMed: 18670423]
59. Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr.* 2004; 24:481–509. [PubMed: 15189129]

60. Essex DW, Li M. Redox control of platelet aggregation. *Biochemistry*. 2003; 42:129–36. [PubMed: 12515547]
61. Jonas CR, Ziegler TR, Gu LH, Jones DP. Extracellular thiol/disulfide redox state affects proliferation rate in a human colon carcinoma (Caco2) cell line. *Free Radic Biol Med*. 2002; 33:1499–506. [PubMed: 12446207]
62. Mieyal JJ, Gallogly MM, Qanungo S, Sabens EA, Shelton MD. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxid Redox Signal*. 2008; 10:1941–88. [PubMed: 18774901]
63. Bellomo G, Mirabelli F, Vairetti M, Iosi F, Malorni W. Cytoskeleton as a target in menadione-induced oxidative stress in cultured mammalian cells. I. Biochemical and immunocytochemical features. *J Cell Physiol*. 1990; 143:118–28. [PubMed: 2318902]
64. Farah ME, Amberg DC. Conserved actin cysteine residues are oxidative stress sensors that can regulate cell death in yeast. *Mol Biol Cell*. 2007; 18:1359–65. [PubMed: 17287397]
65. al-Baldawi NF, Abercrombie RF. Calcium diffusion coefficient in *Myxicola* axoplasm. *Cell Calcium*. 1995; 17:422–30. [PubMed: 8521456]
66. Kushmerick MJ, Podolsky RJ. Ionic mobility in muscle cells. *Science*. 1969; 166:1297–8. [PubMed: 5350329]
67. Dahm LJ, Jones DP. Clearance of glutathione disulfide from rat mesenteric vasculature. *Toxicol Appl Pharmacol*. 1994; 129:272–82. [PubMed: 7992317]
68. Dahm LJ, Jones DP. Rat jejunum controls luminal thiol-disulfide redox. *J Nutr*. 2000; 130:2739–45. [PubMed: 11053515]
69. Go YM, Gipp JJ, Mulcahy RT, Jones DP. H₂O₂-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1. *J Biol Chem*. 2004; 279:5837–45. [PubMed: 14638694]
70. Go YM, Pohl J, Jones DP. Quantification of redox conditions in the nucleus. *Methods Mol Biol*. 2009; 464:303–17. [PubMed: 18951192]
71. Hansen JM, Go YM, Jones DP. Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu Rev Pharmacol Toxicol*. 2006; 46:215–34. [PubMed: 16402904]
72. Gutscher M, Pauleau AL, Marty L, et al. Real-time imaging of the intracellular glutathione redox potential. *Nat Methods*. 2008; 5:553–9. [PubMed: 18469822]
73. Jin H, Heller DA, Kalbacova M, et al. Detection of single-molecule H₂O₂ signalling from epidermal growth factor receptor using fluorescent single-walled carbon nanotubes. *Nat Nanotechnol*. 5:302–9. [PubMed: 20208549]
74. Lee D, Khaja S, Velasquez-Castano JC, et al. In vivo imaging of hydrogen peroxide with chemiluminescent nanoparticles. *Nat Mater*. 2007; 6:765–9. [PubMed: 17704780]
75. Tribble DL, Aw TY, Jones DP. The pathophysiological significance of lipid peroxidation in oxidative cell injury. *Hepatology*. 1987; 7:377–86. [PubMed: 3549510]
76. Ziegler DM. Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem*. 1985; 54:305–29. [PubMed: 2862840]
77. Jones DP, Liang Y. Measuring the poise of thiol/disulfide couples in vivo. *Free Radic Biol Med*. 2009; 47:1329–38. [PubMed: 19715755]
78. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal*. 2006; 8:1865–79. [PubMed: 16987039]
79. Lonn E, et al. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA*. 2005; 293:1338–47. [PubMed: 15769967]
80. Williams KJ, Fisher EA. Oxidation, lipoproteins, and atherosclerosis: which is wrong, the antioxidants or the theory? *Curr Opin Clin Nutr Metab Care*. 2005; 8:139–46. [PubMed: 15716791]
81. GISSI-Prevenzione trial (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet*. 1999; 354:447–455. [PubMed: 10465168]

82. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med.* 1994; 330:1029–1035. [PubMed: 8127329]
83. Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, Gaziano JM, Ridker PM, Willett W, Peto R. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med.* 1996; 334:1145–1149. [PubMed: 8602179]
84. Collaborative Group of the Primary Prevention Project. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. *Lancet.* 2001; 357:89–95. [PubMed: 11197445]
85. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med.* 2000; 342:154–160. [PubMed: 10639540]
86. Zureik M, Galan P, Bertrais S, Mennen L, Czernichow S, Blacher J, Ducimetière P, Hercberg S. Effects of long-term daily low-dose supplementation with antioxidant vitamins and minerals on structure and function of large arteries. *Arterioscler Thromb Vasc Biol.* 2004; 24:1485–1491. [PubMed: 15217803]
87. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med.* 2001; 30:1191–1212. [PubMed: 11368918]
88. Park Y, Ziegler TR, Gletsu-Miller N, Liang Y, Yu T, Accardi CJ, Jones DP. Postprandial Cysteine/Cystine Redox Potential in Human Plasma Varies With Meal Content of Sulfur Amino Acids. *Journal of Nutrition.* 2010; 140:1–6. [PubMed: 19889811]
89. Jones DP, Park Y, Gletsu-Miller N, Liang Y, Yu T, Accardi CJ, Ziegler TR. Dietary sulfur amino acid effects on fasting plasma cysteine/cystine redox potential in humans. *Nutrition.* in press.
90. Moriarty-Craige SE, Adkison J, Lynn M, Gensler G, Bressler S, Jones DP, Sternberg P Jr. Antioxidant supplements prevent oxidation of cysteine/cystine redox in patients with age-related macular degeneration. *Am J Ophthalmol.* 2005; 140:1020–6. [PubMed: 16376645]
91. Moriarty-Craige SE, Ha KN, Sternberg P Jr, Lynn M, Bressler S, Gensler G, Jones DP. Effects of long-term zinc supplementation on plasma thiol metabolites and redox status in patients with age-related macular degeneration. *Am J Ophthalmol.* 2007; 143:206–11. [PubMed: 17157802]

214,000 Cysteine residues encoded in mammalian genome support many structures and functions in proteins

Fig 1. Cysteine residues are prevalent in proteins and undergo a spectrum of covalent and non-covalent modifications that are used to modify structure and function

The human genome encodes 214,000 unique cysteine (Cys) residues in protein sequences. While many of these Cys are contained within internal protein structures and not accessible to reversible modifications or interactions, Cys is a polar amino acid commonly found on the surface of proteins. In the vicinity of cationic amino acids, Cys can undergo ionization to a thiolate, which readily binds transition metals and is reactive with oxidants and electrophiles. These characteristics are associated with a large number of covalent modifications and ionic interactions which are used in control of protein activity, structure, subcellular distribution, cell signaling, macromolecular interactions and trafficking.

Fig 2. The percentage of total Cys and conserved/evolved Cys in cytoplasmic and mitochondrial ribosomal proteins suggests that a large fraction of Cys has biologic function

A comparison of the percentage of Cys found in ribosomal proteins illustrates the conclusion of Miseta and Csutora [4] that selective pressures kept the Cys content to be about half of that expected from random use of the Cys codon (3.2%). The Cys contents in the cytoplasmic small (A) and large (B) subunits are similar (see total and evolved), with most of the Cys residues showing the characteristic of being conserved through evolution or, upon appearance, being conserved in more complex organisms. In the mitochondrial small subunit (C), the percentage of the Cys (total) is similar to the cytoplasmic subunits but the Cys (evolved) are less well conserved with a slower rate of appearance of conserved/evolved Cys. The large mitochondrial subunit (D) has a lower percentage of Cys (total and evolved). In contrast, the mitochondrial targeting sequences (MTS) for the small (E) and large (F) ribosomal subunits had percentages of Cys similar to that expected from random use of the Cys codon (3.28%, broken horizontal line). MTS have few evolved or conserved Cys, perhaps reflecting a more random 3-dimensional structure. These data underscore the conclusion that Cys residues are used sparingly, and when used, are largely retained within conserved protein elements as expected for functionally important amino acids. Data are from Uniprot; J, *Methanococcus jannaschii*; E, *Escherichia coli*; S, *Saccharomyces cerevisiae*; C, *Caenorhabditis elegans*; A, *Anopheles gambiae*; D, *Drosophila melanogaster*; Z, *Danio rerio*; X, *Xenopus laevis*; M, *Mus musculus*; R, *Rattus norvegicus*; B, *Bos taurus*; H, *Homo sapiens*.

Fig 3. Generalized scheme of thiol/disulfide redox changes during the life cycle of cells with associated functional characteristics

Because the ratio of thiol to disulfide concentration (e.g., GSH/GSSG ratio) does not reflect the correct stoichiometry of a two-electron transfer reaction involving a disulfide, data are given in terms of the redox potential (E_h) calculated using the Nernst equation. By convention, E_h values are the two-electron half-cell reduction potential of the disulfide/thiol couple of the redox pair [87]. For example, for the glutathione disulfide-glutathione redox couple, the reduction half-reaction is $\text{GSSG} + 2\text{H}^+ + 2\text{e}^- \rightarrow 2\text{GSH}$; in shorthand notation, this is $\text{GSSG}, 2\text{H}^+/2\text{GSH}$. In common use in biomedical literature, this is termed GSH/GSSG redox potential. On the right side of the double vertical lines in this figure, A–D represent thioredoxin and GSH/GSSG redox couples and redox changes associated with the life cycle of cells. On the left side of the double lines, changes in E_h of the plasma cysteine/cystine couple and associated physiologic changes in vivo. Highly proliferative cells have a more reduced redox environment than non-differentiating cells by several criteria, including the major thiol-disulfide redox control systems dependent upon A) thioredoxins (Trx-1, Trx-2) and B) GSH. Oxidation or loss of functional Trx-1 or GSH results in growth arrest; more severe conditions activate apoptosis or cause necrosis, similar in proliferating cells. C) Differentiated cells have more positive (oxidized) GSH/GSSG redox status. Mild oxidation signals transcriptional activation of stress genes, including those which enhance antioxidant defenses. More extensive oxidation activates apoptosis or causes necrosis similar to that observed in proliferating cells. D) Activation of apoptosis by any mechanism results in extensive oxidation of the GSH/GSSG couple. This scheme is based largely on studies of mammalian cells in cell culture which have been recently reviewed [21]. A limited number of in vivo correlates are available [3,77]. Studies of rat intestine following nutritional and surgical manipulations show associations between growth indices and redox potential (summarized in [21]). Also, in humans, studies are available to show that plasma thiol/disulfide redox potentials become progressively oxidized with age and are oxidized in association with pathology [77,78]. Manipulations of extracellular redox potential in cell culture using a redox clamp model (Go and Jones, *Methods in Enzymology*, in press) also shows that cells respond to controlled variation of extracellular redox potential with increased proliferation in response to more reduced potential and cell stress signaling in response to more oxidized potential.

Fig 4. Schematic diagram representing the subcellular redox potential (E_h) across a cell
 In this cartoon, the simplified cell is illustrated to have extracellular (plasma/interstitial), cytoplasmic, endoplasmic reticular (ER), nuclear and mitochondrial compartments. In the bottom portion, the approximate E_h according to the scale on the left is plotted as a function of the position across the cell, which corresponds to the horizontal broken line in the cell illustration. This scheme is based upon studies of the subcellular redox potentials of the GSH/GSSG and thioredoxin systems, with inferred values for the nucleus based upon measured high GSH concentrations, low protein glutathionylation and a relatively reduced redox potential of nuclear Trx-1, as summarized in the text.

Fig 5. Major types of cell signaling mechanisms depend upon the three major interconvertible energy currencies of living systems

Biologic systems obtain energy to support life from redox reactions in which the potential energy of oxidation-reduction reactions is harvested to perform work, including osmotic and electrochemical work, thereby creating ionic and molecular concentration gradients across membranes. Such electrochemical gradients are used to synthesize chemicals with high potential energy, such as synthesis of ATP from ADP and inorganic phosphate. Cell signaling as well as metabolic regulation involves control mechanisms dependent upon these energy currencies. These include kinase (ATP and related chemicals), redox (oxidation-reduction reactions) and transmembranal ion movement. Thus, systems biology descriptions of redox signaling must ultimately incorporate redox mechanisms with all of these signaling types.

Fig 6. Orthogonal control of signal transduction systems by redox-sensing mechanisms
 The three major types of signal transduction systems for high-energy chemical signaling (illustrated for kinase signaling), redox signaling and ionic signaling, share common features of having proximal signal generators and distal signal response elements. In each case, the signal generators and response elements are proteins. In some cases, specific Cys residues are obligatory components of the signaling mechanism. These are termed “redox-signaling” Cys in the present article. In most cases, other non-obligatory Cys residues are present. Redox-sensitive Cys of this type are considered “redox-sensing” Cys in the present article. The concept of redox sensing is that covalent or non-covalent modification of these redox-sensing thiols provides means to alter protein activity, protein-protein interaction, translational diffusion and other characteristics which can govern amplitude of signals without fundamentally altering the signaling mechanism. Because cells undergo characteristic changes in redox potential through the life cycle, this means that kinase, redox and ionic signaling mechanisms can be “throttled” by appropriate redox-sensing thiols that respond to those thiol/disulfide redox changes in dividing, non-dividing and apoptotic cells. Similarly, because redox potentials are controlled differently in subcellular compartments, the same signaling mechanism can be differentially controlled by the prevailing local redox environment.

Fig 7. Implications of redox-sensing mechanisms to interpretation of signaling within the organizational structure of cells

This figure is based upon the subcellular compartmentalization of redox status, as described in Fig 4. Evidence for localized signaling complexes raises the possibility that redox-sensing mechanisms could be a fundamental component of the functional organization of cells. In Panel A, the possibility is presented that the redox active components in the cytoplasm are rapidly diffusing so that no cytoplasmic redox gradients occur. In Panel B, two types of localized redox effects are depicted, one being a signaling complex activation associated with the plasma membrane (designated “p”) and the other being localized signal activation in the vicinity of mitochondria due to increased oxidant production by mitochondria (designated “mt”). In the former case, a burst of oxidant could provide a local activation/inactivation mechanism and decrease mobility of signaling components to produce a strong localized signal. In the latter case, mitochondria-derived oxidants cause oxidation within the vicinity of mitochondria. Perinuclear mitochondria could preferentially generate signals targeting nuclear activation mechanisms without affecting plasma membrane signaling mechanisms. Panels C and D represent alternative possibilities which could impact cell functions. In C, the observation that nuclei are more reduced than extracellular space implies that there are regional differences in reduction and oxidation between the compartments. Because cytoplasm represents an intervening diffusion space, the redox potential difference between the nucleus and cell membrane must be limited to the nuclear envelope and plasma membrane or be partially shared across the cytoplasmic space. The total redox potential difference between nuclei and extracellular space could be 120 mV or more. If this is distributed in a radial gradient from the nucleus to the plasma membrane, then proteins undergoing redox-dependent associations with cytoskeletal proteins could have differences in concentration in association with this gradient. A 60 mV radial gradient could support a 10-fold concentration gradient while a 120 mV gradient could support a 100-fold gradient. Thus, if a standing redox gradient exists between the nucleus and the plasma membrane, it could provide a mechanism to direct protein distribution. This could localize signaling as well as enhance efficiency of signaling processes. In D, the possibility for polarized cells to have transcellular redox potential gradients is depicted. In intestines, the apical surface is relatively oxidized compared to the basal surface, and this could serve to provide specificity to signaling or enhance efficiency of signaling processes associated with absorption. In lung, the apical surface is relatively reduced compared to the basal surface, again providing a basis for specialization of signaling functions.