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REDOX-BASED REGULATION OF SIGNAL TRANSDUCTION: PRINCIPLES, PITFALLS, AND PROMISES

Yvonne M. W. Janssen-Heininger[1], **Brooke T. Mossman**[1], **Nicholas H. Heintz**[1], **Henry J. Forman**[2], **Balaraman Kalyanaraman**[3], **Toren Finkel**[4], **Jonathan S. Stamler**[5], **Sue Goo Rhee**[6], and **Albert van der Vliet**[1]

1 *Department of Pathology, University of Vermont Colllege fo Medicine, Burlington, VT*

2 *School of Natural Sciences, University of California, Merced, CA*

3 *Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI*

4 *Cardiology Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD*

5 *Department of Medicine, Duke University Medical Center, Durham, NC*

6 *Institute of Molecular Life Science and Technology, Ewha Women's University, Seoul, South Korea*

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Introduction

Both aerobic and anaerobic metabolism are accompanied by the production of reactive oxygen and/or nitrogen species, and organisms ranging from prokaryotes to mammals have evolved an elaborate and redundant complement of defenses to confer protection against oxidative and nitrosative insults. Compelling data also indicates that oxidants are produced and employed in physiological settings as signaling molecules in control of cell and tissue homeostasis, cell division, migration, contraction, and mediator production [1–4]. Collectively, these signaling oxidants--which include nitric oxide (NO), *S*-nitrosothiols (SNO, in particular S-nitrosoglutathione; GSNO) and hydrogen peroxide (H_2O_2)-- are produced mainly by NADPH-dependent enzymes (NO synthases and NADPH oxidases) whose expression is tightly controlled, compartmentalized and tissue-specific. Signaling by these reactive molecules is mainly carried out by targeted modifications of cysteine residues in proteins, including S-nitrosylation and S-oxidations. This manuscript reviews recent findings--made possible by methodological and technical advances that have allowed detection of oxidation events in proteins--and illuminates their significance. We also attempt to highlight some of the current pitfalls, and approaches needed to advance this important area of biochemical and biomedical research.

Correspondence should be addressed to: Yvonne M. W. Janssen-Heininger, PhD, University of Vermont College of Medicine, Department of Pathology, 89 Beaumont Avenue, HSRF 216A, Burlington, VT 05405, Phone: 802-656-0995, Fax: 802-656-8892, Email: yvonne.janssen@uvm.edu.

Oxidants as modulators of signal transduction

The specificity and selectivity of oxidant molecules is dictated by their chemical reactivity. Signal transduction cascades are rapidly and reversibly activated by specific agonists and stimuli, that transmit precisely regulated, compartmentalized signals, to produce a distinct molecular or biochemical outcome. Oxidants employed in signal transduction in physiological settings must exhibit substrate specificity and produce reversible oxidations. Highly reactive oxidant species such as ozone, hypochlorous acid, peroxynitrite, nitrogen dioxide and the hydroxyl radical, oxidize biomolecules without preference or specificity. Most oxidative modifications caused by these highly reactive oxidants, such as the 3-nitrotyrosine and protein carbonyls are also not easily reduced, and are thus unlikely to represent signaling events. Protein modifications by the aforementioned species may lead to the aberrent activation of signal transduction cascades, often resulting in pathophysiology. By contrast, physiological oxidants –principally NO/SNO, and $O_2^{\cdot -}/H_2O_2$, have been implicated in reversible Cys-based modifications that underlie homeostatic control and diverse biological responses.

The role of NO in smooth muscle biology through its regulatory role in the activation of guanylate cyclase via binding to heme Iron and resultant production of cGMP is well established. However, even in vascular smooth muscle, a significant component of the relaxation response may be cGMP independent. In some vessels and species, the response may even be completely independent of cGMP [5–8], and it has been shown that animals deficient in a SNO-metabolizing enzyme have low vascular resistance ([9], JSS unpublished). By analogy, stringent genetic evidence for a role of NO (per se) in blood pressure control has not been provided. SNOs can signal via cGMP or S-nitrosylation. Taken together, these observations suggest that SNOs play a physiological role in regulation of vessel tone.

A cardinal role for S-nitrosylation in signaling that is independent of cGMP was pointed to early on [10,11]. S-nitrosylation of specific cysteine residues (which will be discussed further below) has been detected in well over 100 proteins of all classes and is arguably the principal mechanism by which NO signals [1]. Much like phosphorylation by kinases, S-nitrosylation by NOSs influences protein activity, protein-protein interactions and protein location. S-nitrosylation thus serves as the prototypic redox-based signal. It is important to note that NO signalling by guanylate cyclase and S-nitrosylation are not necessarily mutually exclusive [12,13]. S-nitrosylation has been implicated in transmitting signals downstream of all classes of receptors, including GPCRs, RTKs, TNF, Toll, glutaminergic and other [1,14–16]--acting locally within subcellular signaling domains as well conveying signals from the cell surface to intracellular compartments, including the mitrochondria and the nucleus (e.g. by modification of transcription factors).

S-nitrosylation exhibits a number of essential features that are prototypic of biological signals and may be useful when considering the possibility that additional reactive oxidants subserve signaling roles: I) temporal regulation on physiological timescales (S-nitrosylation is stimulus-coupled and rapid), II) the existence of motifs within proteins that facilitate S-nitrosylation and/or provide specificity [1,17], III) co-localization of target proteins with a source of NO (e.g. NOS enzyme) [18], IV) reversibility; and V) enzymatic control (e.g. S-nitrosoglutathione; SNO) reductase ([1,19]) controls S-nitrosothiol tone [9,20]). In addition, dysregulation of protein S-nitrosylation is associated with a growing list of diseases, including cystic fibrosis, asthma, heart disease, and neurodegenerative diseases. Therefore, understanding of the regulatory events that control S-nitrosothiol biology has the potential to lead to novel therapeutic opportunities [1].

An accumulating literature is making the case for signaling by H_2O_2. Stimulation of growth factor receptors, toll like receptors (TLR), and cytokine receptors, also leads to the activation

of signaling cascades that are regulated by hydrogen peroxide (H_2O_2) as a result of activation of non-phagocytic oxidases (NOX), or dual oxidase (DUOX) (See Table 1) [18,19,21–24]. The expression of multiple isoforms of these enzymes in a variety of tissues provides evidence that the deliberate production of low levels of oxidants is a feature of many cells [25]. H_2O_2, a small and non-charged molecule that is readily produced and removed following these physiological stimuli, exhibits properties that are ideal for signal transduction [2]. Although it was initially believed that H_2O_2 can freely diffuse across membranes, recent genetic evidence suggests that some membranes are poorly permeable to H_2O_2, and that its transport may be regulated by aquaporin channel proteins [26,27], which also regulates transport of NO [28]. H_2O_2 exerts its functions in signaling by causing reversible amino-acid oxidations, as will be discussed below.

Oxidizable amino acids: Reactive cysteines as redox targets

Amino acids that are targets for reversible oxidation include cysteines with a low pKa sulhydryl group (4–5), which causes unique susceptibility to oxidation compared to the typical pKa of non-reactive cysteines (8.5) [29]. In addition, methionine, tryptophan, and tyrosine residues are also prone to oxidative modification, although the functional impact of those events in physiological signal transduction remains to be established. Regulation of cell signaling via the cysteine group is best established [30,31], and will therefore be the focus of further discussion. Numerous classes of proteins contain free cysteine residues that are highly conserved across species, suggesting regulatory possibilities, beyond structural roles and metal ion coordination. In addition, cysteine residues can be modified through alternative redox-based modifications (SNO, SOH, SSG, S-S) that may enable differential effects on protein function [32–35]. In other words, distinct reversible modifications of cysteines (Figure 1) may lead to unique functional outcomes (Figure 2) as well as provide a mechanism through which differential responsitivity can be achieved. For example, sulfenic acid modification (SOH) (hydroxylation) of a single allosteric cysteine has been shown to elicit effects on structure and function that are distinct from those elicited by mixed disulfide (glutathionylation)[32]. Cysteines are thus believed to serve as molecular switches, capable of processing different redox-based signals into distinct functional responses [36–38].

Recent studies have shown that the sulfinic acid form of 2-cys peroxiredoxins can be reduced back to the sulfhydryl in an ATP-dependent enzyme-(sulfiredoxin, Srx) catalyzed reaction [39], and a reversible thioesterifcation has been reported to alter transcriptional activity [40], raising the possibility that additional Cys modifications may be found to have regulatory roles. Cysteines also are uniquely targeted by alkylating species (such as cyclopentenone prostaglandins, acrolein), hydroxynonenal and avicins (which can be formed endogenously, or encountered through environmental insults [41,42]), and by acylation (which has important roles in localizing proteins to the membranes [43]). While a protein can have many cysteine residues, few are reactive (typically those in the thiolate, S^- form). The pKa of a cysteine is dependent on the charge of adjacent amino acids within the three dimensional conformation or quaternary state. Furthermore, the susceptibility to oxidative modification may be governed by the reactivity of individual oxidants, which can be influenced by structural features of the protein (adjacent amino acids, or by coordination of metal ions). As an example, in hydrophobic regions, the reaction between NO and oxygen is accelerated, leading to enhanced formation of S-nitrosylating species [44]. Protein thiols found in consensus motifs wherein the Cys is adjacent to basic and acid residues or aromatic residues often serve as sites of nitrosylation [1]. In addition, transition metals may catalyze S-nitrosylation [1,31].

S-nitrosylation may be mediated by NO, SNO, or several higher oxides [45]. Forman and colleagues [31] and Tannenbaum [46] emphasize that S-nitrosylation may involve nitrosative chemistry (nitrosation refers to chemical addition of NO^+), but is not restricted to reactions of

NO^+. The term nitrosyl, which is mechanism neutral, aptly describes attachment of the NO moiety to a nucleophilic group or transition metal. S-nitrosylation is therefore preferred terminology in biological systems where chemical mechanism is rarely known. Needless to say, "-ylation" [31] is standard biochemical nomenclature for posttranslational modifications of proteins. Notably, searches of Pubmed with "S-nitrosation" as the key term yielded a small number of hits, whereas almost 100 papers on "S-nitrosylation"--covering major advances in the field—have been published this year alone. Few redox-based signaling functions or fundamental advances were identified using chemical nomenclature for NO^+/nitrosation. Given these considerations, we refer to the NO-associated posttranslational modification of cysteine thiol as S-nitrosylation.

Like NO and SNO, H_2O_2 also oxidizes reactive cysteines, leading to the formation of sulfenic acids (SOH) which can be further oxidized to sulfinic (SO_2H) and sulfonic acids (SO_3H) [47–49]. Unlike NO/SNO, H_2O_2 cannot readily access hydrophobic compartments. Sulfenic acids also are byproducts of S-nitrosylation and targets of S-glutathionylation (PSSG). In particular, S-glutathionylation, the formation of a disulfide between the cysteine of glutathione and a cysteine moiety of a protein (also known as a protein mixed disulfide, or PSSG), is believed to be a protective mechanism that prevents further oxidation to sulfinic and sulfonic acids, which are not readily regenerated [47]. PSSG formation is an inevitable consequence of denitrosylation by GSNO reductase, but constitutes a minor pathway.

Relative importance of NO- or H_2O_2-dependent cysteine oxidations in redox signaling

The relative importance of NO/SNO- or H_2O_2-dependent oxidative modifications in cellular physiology vs. pathophysiology and the interplay of these regulatory oxidations is often denated, but prematurely so. Many years of work have established firmly the role of oxidants in pathophysiology and NO's broad-based signaling function. These are the cornerstones of the field. Moreover, NO biology has enjoyed the use of highly specific inhibitors of enzymes that have uncovered its many physiological roles, whereas specific inhibitors of NADPH oxidases are not yet available. Thusfar, the signaling function of H_2O_2 –physiological vs. stress/adaptive --has been far more difficult to establish, important advances notwithstanding. One plausible reason for this lag may relate to the lack of commonly available technology to detect H_2O_2–based reversible cysteine oxidations methodology with sufficient specificity. The instability, or rapid reversal of these redox changes forms an argument for a signaling role, but of course poses practical problems in detecting them. Therefore, it is much easier to list physiological events regulated by NOSs than by NAPDH oxidases at this time, although evidence to support a role for multiple members of the NOX/Duox family in migration, cell differentiation, and mitogenesis is becoming increasingly apparent [25]. At the present time however, oxidative stress is more often linked to disease than nitrosative stress. The extent to which roles may be reversed is an area of active study. In this regard, both the NO and H_2O_2 systems rely on the principle of Cys-based posttranslational modifications. It is therefore of some surprise that while many reviews on the topic of redox signaling have been published [1,3,31,50,51] most highlight either H_2O_2 or NO selectively; co-existence of both oxidants and their respective chemical reactivities within the same cell is rarely considered.

A general classification scheme has been constructed for cysteine oxidations (including those induced by both NO and H_2O_2) which highlights that S-nitrosylation may be important in signaling functions relevant to cellular physiology and homeostasis, while the formation of sulfenic acids, and S-glutathionylation has been linked to cellular stress and adaptation [1, 52]. Indeed, a number of targets such as caspases, receptors (e.g. alpha-amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA) and NMDA), kinases, G proteins and ion channels have been demonstrated to be S-nitrosylated under physiological conditions [15,53,54]. In diverse cell types, S-nitrosylation can be detected *in situ* under both non-stimulated conditions

and following physiological activation (e.g stimulation of GPCRs to dilate blood vessels or stimulate cardiac contractility [55,56]). These findings suggest a role for S-nitrosylation in homeostatic control. Whether H_2O_2-based oxidative modifications (sulfenic acids and glutathionylation) will be afforded the same status is unclear, although high concentrations of GSH would seem to make it a likely substrate [47]. It remains to fully unraveled, for example, whether incorporation of GSH is an activating mechanism or part of a deactivation cycle (a route to reduce the protein) or both.

It seems likely that in physiological settings different cysteines will be targeted by different oxidative effectors; aforementioned regulation via compartmentalization of either NOX or NOS enzymes and various structural features (hydrophobicity, accessibility of GSH etc), will dictate which oxidative events prevail. Furthermore, as will be described below, regulatory enzymes important in redox control, such as thioredoxin, glutaredoxin, peroxiredoxin, and protein disulfide isomerase, are themselves affected and regulated by both H_2O_2 and NO-based cysteine oxidations. It is of interest to note that S-nitrosylation may be criticaly sensitive to oxygen tension, often being potentiated by hypoxia [15,54,57]. Further, it has been shown that oxidation/reduction (of a group) of thiols may regulate S-nitrosylation by an allosteric mechanism [57]. In aggregate, these observations highlight a need for additional studies to formally test the interplay between H_2O_2 and NO-dependent cysteine oxidations.

Control of S-nitrosothiol and H_2O_2 tone

Control of S-nitrosothiol tone

S-nitrosylation, as mentioned above, has emerged as a ubiquitous posttranslational modification, playing an essential role in NO biology. The evidence that S-nitrosylation serves as a physiological mode of signal transduction stems from a number of key observations. First and foremost, S-nitrosylation of proteins is demonstrably coupled to diverse physiological stimuli. Secondly, S-nitrosylation meets the requirements of a regulatory posttranslational modification, including specificity (it tends to target one or few thiols often found within consensus motifs), reversibility and and enzymatic control. [1,58]. Numerous reaction channels may support S-nitrosylation chemistry in vivo, including the reaction of NO with thiols or thiyl radicals, dimerization of NO (on aromatic residues) and oxidation of NO to a molecule with NO^+ character (followed by a reaction with thiols). Oxidation of NO may be catalyzed by transition metals, oxygen or NO itself; NO and O_2 concentrate in hydrophobic compartments, which enables micellar catalysis and protects the SNO from solvent. Acidic conditions, which occur in microdomains of proteins, lysosomes or the mitochondrial inner space may also promote SNO formation from NO or nitrite (as reviewed in [1,19]). Enzymes other than NOS have been shown to promote S-nitrosylation, employing some of the above-described chemistry, and include ceruloplasmin, membrane-associated mammalian hemoglobin and Ascaris hemoglobin. GSNO reductase, (discussed in detail in the next paragraph), which denitrosylates proteins in equilibrium with GSNO, is a major determinant of steady state levels of S-nitrosylated proteins. S-nitrosylation can also be reversed by specific metal ions, such as Cu^+, the low MW antioxidant, ascorbate (see below) and thioredoxin (Trx) [59,60]; the physiological relevance of the latter is still unclear.

Control of H_2O_2 tone

Peroxiredoxins (Prx) have emerged as critical regulatory systems that quench H_2O_2, and act along catalase and glutathione peroxidase. At least six mammalian Prx have been identified, subdivided into 2-Cys, atypical 2-Cys, and 1-Cys Prx, which exist as homodimers in different subcellular compartments ([50] for review). Prx are highly abundant and are believed to control the H_2O_2 tone. Their over-expression lowers intracellular levels of H_2O_2, and leads to

inhibition of signaling induced by PDGF, TNFα, or ceramide, suggesting that Prx enzymes contribute to signaling by removing H_2O_2 [3,50].

The catalytic mechanism of 2-Cys Prxs is relatively well understood. In the fully reduced state, the peroxidatic cysteine of one subunit of a head-to-tail homodimer is attacked by H_2O_2 to generate sulfenic acid (− SOH), and a conformational change facilitates attack of the resolving cysteine, resulting in an intersubunit disulfide bond. The disulfide reductase, thioredoxin (Trx), then reduces the intersubunit disulfide bond, regenerating reduced dimer. In eukaryotic Prxs, a C-terminal domain stabilizes the $–S_P$-OH moiety, thereby promoting further oxidation of the peroxidatic cysteine to sulfinic acid ($-S_P-O_2H$). This process, which is commonly referred to as “over-oxidation” or “hyper-oxidation”, inactivates the normal catalytic cycle because disulfide bond formation with the resolving cysteine is abolished. Over-oxidation of Prx is believed to occur when H_2O_2 concentrations exceed the capacity of Prx regeneration by the Trx system, and originally was proposed to act as a “floodgate” to promote H_2O_2 signaling [61]. While a small portion of PrxI and II have been reported to be hyper-oxidized in response to physiological stimuli, PrxII dampens signaling through interactions with the PDGF receptor in the absence of hyper-oxidation [62].

Although sulfinic acid residues (SO_2H) were originally thought to represent irreversible cysteine oxidations, Sulfiredoxins (Srx) have been identified as cysteine sulfinyl reductases that can repair the SO_2H group of peroxiredoxin, regenerating active enzyme. Repair of the sulfinyl group supports the concept that the Prx inactivation-reactivation cycle represents a cell signaling loop [50,63–66] (Table 1). This notion is supported by recent studies that show that cell cycle arrest is associated with recruitment of hyper-oxidized PrxII to high molecular weight oligomers, and that recovery is correlated with regeneration of reduced enzyme [67]. It is of significance to note that formation of SO_2H forms are not limited to Prx, and that reactive cysteines in many other proteins can also be targets to “over-oxidation,” which may contribute to the regulation of their function.

The molecular regulation of Prx represents an intriguing area of investigation. Originally these enzymes were thought to be relatively weak scavengers of H_2O_2, but recent work shows that are as active as peroxidases as either catalase or glutathione peroxidase. What distinguishes peroxiredoxins from other peroxidases is their interaction with numerous regulatory proteins, including c-Abl, c-Myc, JNK, and the PDGF receptor [62,68–71]. Little is known about how the oxidation state of Prxs influences protein-protein interactions, but one intriguing possibility is that oxidation state of peroxiredoxins may influence the subcellular location or activity of interacting factors. Linking protein-protein interactions to oxidation state, particularly for those proteins that interact with the C-terminal domains that promote hyper-oxidation, would permit peroxiredoxins to function as peroxide dosimeters in a wide variety of redox-dependent signaling events.

Peroxiredoxins have also have been shown to stack in complex 3-dimensional structures [72]. Prx-1 appears to have a function as a molecular chaperone, which is controlled by its phosphorylation state [73]. Phosphorylation of PrxI and II by cdc2/cyclin B complexes regulates oligomeric transitions and peroxidase activity, providing yet another mechanism to control the activity and structural state of these enzymes during the cell cycle. Changes in Prx mRNA and protein expression levels occur in many disease states, including cancer [74–77], suggesting that control of Prx function is regulated at both transcriptional and post-translational levels. While these observations show that the biological functions of individual Prxs exceed the mere reduction of H_2O_2, additional investigation is needed to define the array of protein complexes that include Prxs in mammalian cells, and to understand how changes in oxidation state and structural transitions of Prxs contribute to signal transduction in specific regulatory networks.

Biochemical and genetic regulation of reversible cysteine oxidations

Reversible cysteine oxidations potentially relevant to signal transduction include disulfides (S-S), sulfenic acid (SOH), glutathionylated cysteines (PSSG), nitrosylated cysteines (SNO) (Figure 1, Table 1). It is important to recognize that steady state levels of these oxidized moieties appear to be controlled directly or indirectly by a number of enzyme systems that control unique redox modules within cells (Table 1). It is notable that enzymes specifically metabolize superoxide (O_2^-), H_2O_2, and GSNO. No mammalian enzyme has been shown to metabolize NO.

GSNO reductase, also known as glutathione-dependent formaldehyde dehydrogenase, breaks down S-nitrosoglutathione. GSNO is converted to GSNHOH, ultimately forming ammonia (NH_3) and glutathione disulfide (GSSG) [78]. GSNO-reductase is highly specific for GSNO and thus regulates the steady state levels of S-nitrosylated proteins that are in equilibrium with GSH [78,79]. Mice deficient in GSNOR have low vascular and airway resistance, and increased cardiac ouput. Intriguingly, the activity of GSNO reductase had been demonstrated to by upregulated in a mouse model of asthma, mediating decreases in lung S-nitrosothiol levels, and increases in airway hyperresponsiveness (AHR), a cardinal feature of asthma. GSNO reductase knock-out mice had elevated levels of lung S-nitrosothiols, which act as endogenous bronchodilators and protected against increases in AHR or tachyphylaxis [20]. GSNO reductase knock-out mice were also hypotensive under anesthesia and showed enhanced susceptibility to mortality from endotoxin or septic shock [9]. GSNO reductase activity also has been linked to β-adrenergic receptor signaling via its regulation of S-nitrosylation state of G-protein coupled receptor kinase-2 [80], a central mediator of GPCR densensitization. Intriguingly, polymorphisms in the GSNO reductase gene were recently identified and demonstrated correlations with asthma susceptibility [81]. Of great interest, the knockout mice also exhibit insulin resistance [82] and show a unique signature of S-nitrosylated proteins: GSK-3-β, AKT and IRS are excessively S-nitrosylated well explaining the phenotype. In aggregate these findings illuminate the functional significance of the GSNO reductase system in general and protein S-nitrosylation by GSNO in particular, and they establish dispositively the role of S-nitrosothiols in the control of cardiac, metabolic, vascular, and respiratory function.

The disulfide reductases, thioredoxin (Trx) and glutaredoxin (Grx) play a major regulatory role in controlling redox homeostasis (Table 1). Both Trx and Grx contain a Trx fold and a common dithiol/disulfide active site motif (Cys-X-X-Cys, [83], for review). Trx catalyze the reversible reduction of disulfides, resulting in a reduced pool of dithiol target protein, and a disulfide in the active form of Trx which is then reduced by thioredoxin reductase, using electrons from NADPH. Two mammalian Trx systems exist: the cytosolic Trx1/TrxR1, and mitochondrial Trx2/TrxR2. A number of proteins have been identified to be redox regulated by Trx and include Nuclear Factor kappa B, (NF-κB), Activator Protein-1 (AP-1), p53, glucocorticoid and estrogen receptors, and HIF1α [84–89]. Trx1 is ubiquitously expressed and is upregulated under inflammatory conditions [83]. Intriguingly, following certain stimuli, Trx1 can also be translocated to the nucleus [90], or to the extracellular compartment following leaderless export [91]. Circulating levels of Trx were reported to be increased in patients with heart disease, rheumatoid arthritis, acute lung injury, or HIV infection, and showed various associations with severity of disease [92–95]. Extracellular Trx can act as a unique chemoattractant for neutrophils, monocytes, and T cells [96]. A 10-kDa C-terminally truncated variant of Trx (termed Trx80) is also secreted and present in plasma, and acts as a potent mitogenic cytokine that stimulates growth of resting human peripheral blood mononuclear cells [95], and differentiation of human CD14(+) monocytes into a novel phenotype in association with activation of mitogen activated protein kinases [97]. The use of a kinetic trapping technique to identify targets that bind extracellular Trx1, has unraveled a highly specific redox-based

interaction between Trx1 and tumor necrosis factor receptor superfamily member 8 (also known as CD30), and that this interaction is important in the regulation of lymphocyte effector function [98].

Mammalian cells also contain glutaredoxins (Grx, also known as thiol transferases), which under physiological conditions act to reverse S-glutathionylated cysteines, restoring the protein cysteine to the sulfhydryl group ([47,83,99], for reviews). The Grx system consists of Grx enzymes, GSH, glutathione reductase, and NADPH. Grx carry out de-glutationylation reactions, also known as the reduction of protein-mixed disulfides (PSSG, glutathionylated cysteines) which requires the N-terminally located active site cysteine in Grx. The reduction of PSSG by Grx results in a mixed disulfide between GSH and the N-terminal active site cysteine (glutathionylation of Grx) which is then reduced by a second GSH molecule. Glutathione disulfide (GSSG) is finally regenerated by glutathione reductase at the expense of NADPH [47,83,99]. Although the major enzymatic action of mammalian Grx, under physiological conditions has been linked to de-glutathionylation of protein targets [47,100], it should be highlighted that under conditions of oxidative stress or damage, where GSSG levels are increased, Grx causes increases in protein S-glutathionylation, instead of decreases [83]. In addition to the monothiol reduction of glutathionyl disulfides, bacterial Grx also reduce low molecular weight disulfides, or disulfides in ribonucleotide reductase through a dithiol mechanism [83,99]. However for mammalian Grx, substrate specificity towards S-glutathionylated proteins has been demonstrated [101]. It is of interest to note that sulfiredoxin also was recently demonstrated to de-glutathionylate protein targets such as actin and protein tyrosine phosphatase 1B [102]. Multiple Grx genes are known in plants and in pro- and eukaryotes; in mammalian cells, Grx1 has been described and localized in the cytoplasm. In addition, Grx2 which has 1.5–3 fold higher catalytic efficiency than Grx1, is localized in mitochondria [47]. Steady state levels of Grx itself appear to be dynamically regulated, and increases in Grx occur in cancer and cardiovascular disease [83,103]. Elevated levels of S-glutathionylated proteins in serum have been suggested to represent a risk-marker for arteriosclerosis obliterans [104]. Recent studies from our laboratory (Y J-H) have demonstrated that multiple stimuli, such as the TLR ligands, lipopolysaccharide (LPS) and CpG DNA, interleukin-4, and transforming growth factor-beta 1 (TGF-β1) result in decreases in Grx activity in primary lung epithelial cells, in association with decreases in Grx1 mRNA, in response to TGF-β1 [105]. In contrast, increases in Grx activity occurred in cells exposed to Interferon-gamma (IFN-γ), in association with increases in Grx1 mRNA, and increases in Grx activity and Grx1 mRNA were observed in mice with allergic airways disease [105].

It is of interest to note that both Grx1 and 2 can be S-nitrosylated, in association with the formation of one disulfide bridge in the active site, and nitrosylation of three structural cysteines. S-nitrosylation resulted in decreased activity of Grx [106]. Furthermore, Trx [107, 108] and protein disulfide isomerase (PDI) (see below) are also targets for S-nitrosylation. Intriguingly, in addition to Grx itself, Trx and PDI are targets for S-glutathionylation [109, 110]. S-glutathionylation of Trx at cysteine 72, abolished the disulfide reductase activity of Trx [109], whereas S-nitrosylation of cysteine 63 was reported to increase Trx activity [107]. S-nitrosylation of cysteine 72 has been recently implicated in transnitrosation of caspases, but did not seem to affect Trx activity [111,112]. Interestingly Trx may also impact S-nitrosothiol homeostasis via its ability to cleave GSNO and protein SNO [60,113]. These collective observations point to important cross regulation of the various redox systems which may have impact on steady state levels of various cysteine oxidation states controlled by these systems.

The aforementioned enzyme systems, operate together with classical antioxidant defenses consisting of superoxide dismutases, catalase or glutathione peroxidases to control steady state levels and/or molecular actions of superoxide anion ($O_2\cdot$), H_2O_2, NO, and SNO. Thus the notion is emerging that cells have evolved with multiple and independently regulated systems,

consisting of cysteine/cystine, GSH/GSSG, and Trx-SH/Trx-SS to control the redox tone [114], a concept that also warrants inclusion of measurements of NO/SNO homeostasis (nitroso-redox homeostasis). Furthermore, the existence of multiple reversible cysteine modifications in proteins warrants a redefinition of the general concept of "oxidative and nitrosative stresses" to include the identity of particular oxidative event (culprit protein, Cys identity, oxidative or nitrosative modification), and its contribution to cellular or tissue (patho-) physiology. Because of the existence of independent regulatory systems, it has become apparent that concentrations of SNOs or H_2O_2 are kept under tight control to permit NO- or H_2O_2-induced signaling events in spatially and temporally controlled settings.

The impact of cysteine oxidations on cell signaling

A number of exciting cysteine targets in proteins have been identified and numerous reports have revealed that cysteine oxidation events can have important functional consequences for an array of signal transduction cascades. It has become apparent that multiple classes of regulatory proteins are reversibly oxidized by H_2O_2 or NO/SNO, and that these oxidation events also have broad implications. The full breadth of S-nitrosylation is beyond the scope of the current review; excellent examples exist for every major class of protein. In many of these cases, the evidence for physiological relevance includes, dependence (of S-nitrosylation-regulated activity) on NOSs, identification of SNO-protein *in vivo*, recapitulation of SNO effects with NO donors *in vitro*, and dominant-negative effects of Cys mutation (e.g. see [115]). Evidence of this sort has not yet emerged generally for H_2O_2 signaling. However, a few good examples may be paradigmatic of what the future holds. The occurance of cysteine modifications in multiple classes of proteins with signaling roles, and their functional impact will be discussed in the following paragraphs. It should be noted that many of these proteins have also been shown to be S-nitrosylated.

Regulation of tyrosine phosphatases

Analogies between phosphorylation and S-nitrosylation were made in the early 1990s [10, 11]. The first studies to broaden this idea beyond S-nitroylation to include other Cys oxidations centered around protein tyrosine phosphatases (PTPs, as reviewed in [17,50]). The first demonstration that PTP is reversibly oxidized by H_2O_2 produced in response to EGF was reported in 1998 [116]. These enzymes which catalyze the dephosphorylation events on tyrosine residues are unique in that they possess a reactive cysteine (pKa= 4.7–5.4) in the catalytic domain which is required for enzymatic activity. Oxidation of this reactive cysteine leads to inactivation of the phosphatase (Figure 2, box 1), and results in an overall increase in tyrosine phosphorylation events, within a specific signaling module. Inactivation of tyrosine phosphatases by H_2O_2 has been demonstrated in a number of settings, including physiological scenarios, such as stimulation of cells with the growth factor, epidermal growth factor, (EGF), or PDGF [17]. Reversible inactivation of the tyrosine phosphatases, PTP-1B [116], PTEN [117], and SHP-2 [29] also have been reported, and a formation of a sulfenic acid intermediate was reported as the oxidative event, although the formation of sulfenyl amide species (Cys-S-N-R) and glutathionylated species also have been shown [118,119]. Elegant biochemical analyses were done to demonstrate the sulfenic and sulfenyl amide forms of PTP1B *in vitro*, and the glutathionylated form of PTP1B has been shown in intact cells. Indeed, in stimulated macrophages that produce H_2O_2 on the outside of the cell, H_2O_2-dependent PTP1B glutathionylation occurs, although the rate constant for formation of the sulfenic acid intermediate suggests that it would not be the intermediate [120,121]. PTP1B, PTP, PTEN and SHP2 are also substrates for inhibitory S-nitrosylation [1,122–127], and singlet oxygen also inactivates protein tyrosine phosphatase-1B by oxidation of the active site cysteine [128], demonstrating that this class of proteases is a target for inhibition via multiple cysteine oxidations.

Oxidative inactivation of PTPs, coupled to the observations that tyrosine phosphorylation requires H_2O_2 production, suggests that activation of receptor tyrosine kinases per se following binding of growth factor may not be sufficient to increase steady state levels of protein tyrosine phosphorylation, and that concurrent inactivation of PTPs by H_2O_2 is needed [50]. Mitogen activated protein kinase phosphatases (MKP) also have a reactive cysteine, required for catalytic activity, and oxidation of the cysteine in MKPs result in inactivation of the phosphatase activity, leading to enhanced activation of MAPKs, including JNK and ERK [129–131]. The enhanced activation of protein kinases as a result of inactivation of the phosphatases represents one mechanism whereby H_2O_2-associated oxidations enhance signaling by RTKs. In contrast to earlier reports, the stress-inducible MKP, Sdp1 in yeast was recently demonstrated to acquire enhanced catalytic activity under oxidizing conditions via the formation of an intramolecular disulfide bridge which in coordination with an invariant histidine side chain facilitates recognition of tyrosine-phosphorylated MAPK, raising the potential that this pathway may be critical in adaptive responses to oxidative stress [132]. Whether similar MKPs that can be activated through oxidative mechanisms also exist in mammalian cells remains to be identified.

Regulation of proteases

Proteases such as caspases and matrix metalloproteinases are another example of enzyme-regulation by oxidative events. Caspases contain an active site cysteine and a role for NO in preventing caspase activation has been established based upon studies demonstrating that mitochondrial caspase 3 and 9 are S-nitrosylated under basal conditions. In response to a pro-apoptotic stimulus, such as Fas ligand of tumor necrosis factor-α (TNFα), caspase denitrosylation occurs in association with activation and subsequent execution of the apoptotic pathway [53,133](Figure 2, box 1). Intriguingly, it has been suggested that Trx (Cys 73-SNO) may exert an antiapoptotic effect by trans-nitrosylation of pro-caspase 3 [111,112]. In addition, H_2O_2 has been shown to inhibit the activation of caspases [134]. A recent study demonstrated that caspase-3 activation during TNFα-induced apoptosis was negatively regulated by S-glutathionylation, and could be reversed through the thiol transferase activity of Grx [135]. Not suprisingly, caspase activation can also be negatively regulated by direct cysteine alkylation with biologically generated electrophiles, such as acrolein or hydroxynonenal [42, 136].

In contrast to the oxidant sensitivity of critical cysteines in the family of caspases, the metzincin metalloproteinase superfamily, which include matrix metalloproteinases (MMPs) and a disintegrin and metalloproteases (ADAMs), contain an invariant cysteine residue in their prodomain region, which maintains latency by chelating the active site zinc center, and activation of these proteases requires disruption of this Cys-Zn interaction (the cysteine switch). Various studies have suggested that MMPs and ADAMs can be activated by oxidants or by NO/SNO, which is suggested to involve oxidation and/or S-nitrosylation of the prodomain cysteine residue [137–139], although this is generally supported by indirect evidence. Biochemical analysis of oxidative activation of purified MMPs has shown that NO or H_2O_2 are ineffective at directly activating these proteases, but they can be activated by more potent oxidants such as HOCl or $ONOO^-$ [140,141]. However, since these oxidants also have the capacity to inactivate MMPs [140,142,143], and MMP activation is thought to occur primarily extracellularly after secretion, it is still unclear to what extent redox signaling events by NOX or NOS regulate MMP/ADAM activation.

Regulation of molecular adaptors and chaperones

Sophisticated systems of molecular chaperones have evolved that ascertain correct folding of newly synthesized proteins and prevent misfolding or protein aggregation. These protein systems consisting of the family of heat shock proteins (Hsp) and protein disulfide isomerase

(PDI) are operative within the cytoplasm and endoplasmic reticulum, respectively [144]. Hsp and PDI also play a role in physiological signal transduction as they are part of signaling scaffolds, and control transport of S-nitrosothiols, respectively (see below). For instance, Hsp90 is essential for the activation of NF-κB, and is a part of the NF-κB signalsome [145, 146]. Because the molecular actions of Hsp and PDI rely on reactive cysteines, they are emerging as potential regulators of redox signaling. The cytosol and the endoplasmic reticulum (ER) have unique redox and ionic milieus, the cytosol (reducing) and the ER (more oxidizing), requiring distinct chaperone networks [36]. Studies initially conducted with Hsp33, which is present in prokaryotes and some eukaryotes, revealed that activation of some Hsp is linked to redox changes. Hsp33 in its inactive conformation has four reactive cysteine residues in the thiolate anion form that bind Zn^{2+}. Under conditions of oxidative stress, the four thiolates form two intramolecular disulfide bonds which cause release of Zn, and subsequent dimerization of oxidized monomers, which effectuates full activation of Hsp33's function as a chaperone [147–149]. Similarly, mammalian Hsp25, 60, 70, and 90 also have redox active cysteine residues, and their oxidation has been linked to the modification of various chaperone functions in conditions of oxidative stress [150–153]. Various cysteine oxidations have been linked to Hsp activation that include nitrosylation, glutathionylation, and disulfide formation [151, 152,154,155] (Figure 2, box 3). Hsp90 is, of note, regulated by S-nitrosylation [155].

PDI is a multi-domain, multi-functional member of the thioredoxin superfamily that contains two thioredoxin-like catalytic domains. PDI can catalyze thiol-disulfide oxidation, reduction and isomerization [156]. The PDI family comprises PDI and PDI-like proteins of which more than a dozen human members have been characterized [156]. PDI is mainly is present in the lumen of the endoplasmic reticulum ER of eukaryotic cells, where many folding factors and protein chaperones are co-located. PDI is oxidized by the ER oxidoreduction proteins Ero1-Lα and Ero1-Lβ in mammalian cells. Oxidized PDI, with active-site cysteines in the disulfide form, can act as an electron acceptor to form disulfide bonds in sulfhydryl-containing substrate proteins [157]. PDI is believed to act as a redox driven chaperone [158], although other reports have questioned the general concept of redox-regulated chaperone activity of PDI [159]. PDI is also surface associated, where it may play a role in transport of NO from S-nitrosothiols across membranes (whether directly or by maintaining surfact thiols in the reduced state is not clear) [160,161]. Interestingly, S-nitrosylation of PDI [162,163] has been linked to neurodegenerative disease [162]. Interestingly, PDI itself can mediate its own denitrosylation [163].

The transduction of molecular signals from receptors that themselves do not have kinase activity relies on the assembly of adaptor proteins in molecular scaffolds to recruit downstream kinases. The carefully timed assembly of these specialized "signalsomes" in precise cellular compartments ascertains the timing and specificity of downsteam responses [164,165]. As an example, stimulation of tumor necrosis factor receptor-1 (TNF-R1) can lead to multiple outcomes, which depends on the differential presence or recruitment of adaptor proteins into the signalsome. While TRAF-2 is required for the activation of JNK, FADD is required for caspase activation, and RIP and Hsp90 are important for the activation of NF-κB. TRAF2 can bind to and activate apoptosis signal-regulating kinase (ASK1), an important regulator of apoptosis [166,167]. Through a genetic screen, Trx was identified as an interacting partner of ASK1, and was demonstrated to regulate ASK1 function via a redox mechanism. The interaction between Trx and ASK1 was found to be highly dependent on the redox status of Trx. Reduced Trx binds ASK1 and keeps the enzyme inactive, suggesting that Trx is a physiological inhibitor of ASK1. Oxidation of Trx1, involving the formation of an intramolecular disulfide between cysteines 32 and 35 resulted in release of Trx1 from ASK1 which results in activation of ASK1 [167]. This redox dependent pathway from Trx to JNK has been demonstrated to occur in macrophages that were stimulated to produce H_2O_2 [168]. However, others reported redox independent regulation of ASK1 by Trx [169]. Trx is subject

to S-nitrosylation at multiple sites. S-nitrosylation of cysteine 69 that lies outside the active site occurs under basal conditions, facilitates oxidoreductase activity, and is associated with protection of apoptosis [107]. Additional reports that S-nitrosylated Trx specifically transnitrosylates pro-caspase 3 [111,112] underscore the functional importance of Trx in protection against apoptosis. In contrast, S-nitrosylation of active site site cysteines (32 and/or 35) promotes disruption of ASK1 and is associated with enhanced apoptosis [108]. In lung epithelial cells, exposure to H_2O_2, or expression of NOX1 disrupted the signaling through TNF-R1, leading to preferential and prolonged activation of JNK, while preventing the activation of IKK, and NF-κB. These events were associated with degradation and cleavage of RIP and Hsp90, repectively, two adaptor proteins required for the activation of IKK [170]. Further, H_2O_2 resulted in recruitment of TRAF2 and ASK1 to TNF-R1, which were functionally important in the activation of JNK and subsequently cell death. Collectively, these data confirm that oxidant-based signals utilize a TNF-R1-ASK-1JNK signaling axis to trigger cell death, and prevent activation of NF-κB [171].

The Nrf2-KEAP system provides another example of the functional significance of redox regulation of cellular adaptors. Nrf2-KEAP acts as a regulatory system that protects cells from an array of stresses. When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, the transcription factor Nrf2 activates antioxidant responsive element (ARE)-dependent gene expression to maintain cellular redox homeostasis. Because of its role in activation of genes with diverse functions, such as antioxidant and phase II enzymes, Nrf2 is now recognized as a key regulatory factor that controls expression of an array of genes important in the defense against diverse environmental insults, a topic that has been extensively reviewed, including in this journal [172,173]. Nrf2 activation is prevented by binding to KEAP-1. Oxidative insults target KEAP-1 by targeting cysteines 273 and 288, which cause dissociation of KEAP-1 from Nrf2, allowing accumulation of Nrf2 in the nucleus and activation of target genes [174,175]. Investigations into the mechanisms whereby KEAP-1 promotes degradation of Nrf2 have unraveled that KEAP-1 acts as a redox sensitive adaptor for a Cul3-based E3 ligase [176]. Thus, under homeostatic conditions, the complex of KEAP-1, Nrf2 and Cul3 ascertains rapid degradation of Nrf2 via the ubiquitin proteasome system, while under conditions of oxidative stress the associations between KEAP-1 Nrf2 and Cul2 are no longer intact, thereby preventing degradation of Nrf2 [177]. Importantly a somatic mutation of the KEAP-1 gene has been identified in lung cancer leading to a glycine to cysteine substitution in the DGR domain, which reduces KEAP-1's affinity for Nrf2, providing a structural basis for the loss of KEAP-1 function and gain of Nrf2 function [178,179]. These findings convincingly illustrate the importance of oxidation events in adaptor proteins in controlling redox signaling, and also that a disturbance in these interactions is associated with human disease.

Genetic screening for protein-protein interactions has identified that S-nitrosylation regulates protein-protein interactions, which include interactions of NOS with various substrates for S-nitrosylation [180]. These findings highlight the compartmentalized and targeted actions of NOS enzymes, and their specific S-nitrosylation of effector proteins that include NMDA and ryanodine receptor, G-protein coupled receptor kinase 2, caspases, GAPDH, NSF, dynamin, cycooxygenase 2 [1,58,80], and many other protein targets. S-nitrosylation also is known to affect the function of molecular chaperones, and this paradigm plays a central role in the regulation of apoptosis. Although the previous paragraph highlighted the significance of caspase S-nitrosylation in the prevention of apoptosis, a wealth of information has also accumulated in support of a pro-apoptotic role in settings where high levels of NO are present. This role of NO in cell death had been associated with formation of highly reactive oxidants, such as peroxynitrite ($ONOO^-$) [181,182]. However elegant recent studies demonstrate that in settings of high NO output, S-nitrosylation in fact plays a central role in apoptosis [183]. In the latter scenario, glyceraldehyde-3-dehydrogenase (GADPH) is the target of S-nitrosylation

of catalytic site cysteine 150, which in turn causes binding of the E3 ligase, Siah1 to GAPDH, and stabilizes Siah1, which is otherwise rapidly turned over. The SNO-GAPDH-Siah1 complex can then translocate to the nucleus, where Siah1 targets nuclear proteins for degradation [184,185].

Redox control through functional dimerization of redox enzymes with signaling intermediates

A number of studies recently emerged to support the notion that enzymes involved in redox homeostasis that include Trx, and its homologs, glutathione peroxidase, and glutathione S-transferase (GST), play a role in redox signaling through redox dependent interaction with a protein with signaling function. Perhaps the best known example of this scenario represents the interaction of Trx with its target proteins, including (ASK-1, Trx binding protein-2, and Trx interacting protein [83]. Oxidation of Trx results in a conformational change that changes interaction with the partner protein, leading to a signaling event (Figure 2, Box 4). Well described is the Trx1-oxidation dependent dissociation of Trx1 from ASK-1 which is critical to the activation of JNK [167] that was described earlier.

A recent study identified nucleoredoxin (Nrx), a member of the Trx family, as a critical regulator of Wnt/β-catenin, signaling pathway evolutionary conserved from nematodes to mammals. Specifically the authors demonstrated that Nrx suppresses Wnt-β-catenin, by redox-dependent binding to Dishevelled, and masking its basic PZD domain, which prevents activation of the pathway. Upon oxidation of reactive cysteines of Nrx1, its association with Dishevelled is lost which promotes stabilization of β-catenin and subsequent activation of the transcription factor T cell factor (TCF). In support of the redox dependence of the Dishevelled-Nrx1 interaction, exposure to H_2O_2 also resulted in disruption of the complex, leading to subsequent accumulation of β-catenin and activation of TCF [186]. It is worthwhile to mention that these conclusions were reached based upon mutagenesis experiments that encompassed mutations of cysteines 205 and 208 of Nrx, and that direct evidence for oxidation of those sites was not provided.

The Yap1 transcription factor in yeast S. cerevisiae regulates hydroperoxide homeostasis, and its activity is increased under conditions of oxidative stress. Intriguingly, redox control of Yap1 is not exerted through oxidation of Yap1 itself, but via glutathione peroxidase (Gpx)-like enzyme, Gpx3, which acts as the sensor and tranducer of the redox signals. Oxidation of cysteine 36 of Gpx3 resulted in an intermolecular disulfide bond with cysteine 598 of Yap-1, and the resolution of this intermolecular disulfide bond into a Yap1 intramolecular disulfide bond, causes activation of the regulator [187].

Glutathione S-transferase pi (GSTp) has emerged as a critical regulator of the JNK pathway. GSTp was demonstrated to be directly associated with JNK [188] and its monomeric form was shown to inhibit JNK activation in a manner that was independent on MEKK1-MKK4, the physiological upstream activators of JNK. In response to an oxidant stress, GSTp oligomerizes, and dissociates from JNK1, promoting its activation [189] (Figure 2, box 4). Importantly, mouse embryo fibroblasts from GSTp-null mice exhibited a high basal level of JNK activity, [189] and GSTp-null mice showed a phenotype of increased myeloproliferation, in association with activation of JNK and the JAK/STAT pathway [190], corroborating the functional significance of GSTp in the regulation of JNK, and potential other pathways *in vivo*. GSTp also is an important regulator of peroxiredoxin 6 (1-cysPrx), via a mechanism that involves heterodimerization of the oxidized catalytic cysteine of 1-cysPrx with pi GST followed by its GSH-mediated reduction and enzyme activation [191].

Redox regulation of transcription factors

In addition to redox control through oxidation of kinases and phosphatases, molecular adaptors, or chaperones, that can in turn regulate the activity of transcription factors, transcription factors themselves can also be direct targets of redox control, a topic that has been extensively reviewed [192]. Perhaps the first evidence for this was based upon the work on the bacterial transcription factor OxyR which was demonstrated to be directly activated by oxidants via the formation of a reversible disulfide bond [193–195] (Figure 2, box 5). Subsequent work has shown that S-nitrosylation, S-glutathionylation, and S-hydroxylation can also activate OxyR [32,196]. This paradigm has been expanded; multiple families of transcription factors in mammalian cells that include NF-κB, AP-1, p53, HIF1α, and HSF are known to be modulated directly through oxidative changes. In many cases, critical reactive cysteines have been identified, which upon oxidation elicit a functional change either manifested as increased transactivation or inhibition. Examples of transcription factors that are inhibited following cysteine oxidation include the p50 and RelA (p65) subunits of NF-κB, and the c-Jun member of the AP-1 family. Further, both S-nitrosylation and S-glutathionylation of those proteins has been shown to interfere with their DNA binding [197–201], and S-nitrosylation is coupled to iNOS function *in vivo*. Multiple cysteines in murine p53 were found to be functionally important, and their selective mutation afftected various aspects of its function [202]. S-glutathionylation of cysteine 82 of p53 has been linked to decreased binding to DNA in association with the formation of monomers and high MW oligomers [203]. Interestingly, the redox state of cysteine 277 of human p53 has been linked to its ability to bind to bind p53 response elements in a sequence specific manner [204]. Hypoxia-inducible factor (HIF)1α also is regulated in a redox dependent manner, and its degradation and activation are regulated via cysteine oxidation. S-nitrosylation of HIF1α promotes its stabilization [205], and S-nitrosylation of Cys-800 activates HIF1α-p300 interaction and stimulates protein transactivation [206].

The importance of oxidation of Hsp in redox signaling was discussed in the previous paragraph. Interestingly, mammalian heat shock factor 1 (HSF1), a transcription factor critical in the induction of stress-inducible Hsp gene expression is directly activated by oxidant stress. Activation of mammalian Hsp gene expression by HSF1 requires conversion of its monomeric state to the DNA-binding competent homotrimer. The sensing of both stresses requires two cysteine residues within the HSF1 DNA-binding domain that are engaged in redox-sensitive disulfide bonds. HSF1 derivatives in which either or both cysteines were mutated are defective in stress-inducible trimerization and DNA binding, stress-inducible nuclear translocation, and Hsp gene trans-activation, and in the protection of mouse cells from stress-induced apoptosis [207].

An emerging body of evidence is accumulating to support the concept that alterations in the activities of transcription factors may not limited to their direct oxidative modification. Indeed chromatin remodeling factors, such as histone acyl transferases (HAT), or histone deacetylases (HDAC) also can sense redox changes, and thereby lead to changes in transcription. The sirtuin (sirt) family of nicotinamide adenine dinucleotide-dependant (NAD) deacetylases plays an important role in aging and metabolic regulation via control of the FOXO family of Forkhead transcription factors [208]. In response to oxidative stress, sirt1 forms a complex with FOXO3, leading to sirt1-dependent deacetylation of FOXO3, which mediates cell cycle arrest, resistance to oxidative stress, and inhibition of cell death [208]. Other reports corroborate a link between oxidative stress and sirt-dependent deacetylation of FOXO, subsequent enhanced activity of FOXO as a transcription factor, and cellular protection against oxidative stress, due to transcriptional upregulation of protective genes such as manganese superoxide dismutase and growth arrest and DNA damage inducible (GADD)45 [209,210]. In contrast, it has been reported that H_2O_2 resulted in acetylation of FOXO4 in association with decreases in transcriptional activity (although the same study also reported that H_2O_2 stimulates binding of

sirt1 to acetylated FOXO4 [211]). Taken together, these data raise the possibility that FOXO4 transcription is regulated in a bi-phasic manner. Notably in this regard, mild transgenic overexpression of sirt1 in mouse hearts was associated with protection against oxidative stress, and ageing, while high overexpressors displayed enhanced cardiomyopathy [212]. In addition to regulation of the sirtuin class of histone deacetylases by oxidative stress, HDACs linked to transcriptional activities of NF-kB, and AP-1 [213–215]) may be inhibited by H_2O_2 (or environmental stresses), and a role for S-nitrosylation has likewise been identified with HDACs in the context of CREB induced gene expression [216].

While all these observations provide compelling links between H_2O_2, changes in histone acetylation and, enhanced transcriptional activity, some of these studies examined conditions of overt stress, characterized by decreases in levels of GSH, increases in GSSG, and evidence of tyrosine nitration [214,215], and highlight the need for additional studies to understand redox regulation of chromatin remodeling factors under physiological settings, which also should entail identification of target amino acids that are oxidized.

Criteria for Signaling: SNO and more

The pharmacological tools in NO biology, including mutant animals (eNOS, nNOS, eNOS, GSNOR) and multiple classes of highly specific NOS inhibitors have established beyond doubt NO's broad-based signaling function in physiology and disease. Further, compelling data revealing the presence of S-nitrosylated proteins by multiple techniques –chemical assays, SNO antibodies, mercury-coupled photolysis chemiluminescence —have helped to firmly establish S-nitrosylation as an omnipresent signal. Among the various posttranslational modifications, phosphorylation, ubiquitylation and S-nitrosylation, are truly ubiquitous. By contrast, pharmacological tools such as inhibitors or NADPH oxidases, and NOX knock out animals are not completely available and alternative cysteine oxidations have been diffcult to assay. Many of the current research findings, particularly as they relate to glutathionylation and sulfenic acid oxidations are limited to test tube settings. In addition, whereas the assays for SNO are relatively specific, those for alternative oxidations do not readily differentiate SNO, SOH or SS. Consequently the functional importance of individual cysteine oxidations in homeostasis or pathophysiology remains largely unknown; the physiological significance of redox signaling at large is, therefore, still an open question. More specifically, the stringent demonstration of agonist-coupled, reversible modifcations of Cys residues by glutathionylation or hydroxylation that subserves diverse functions or that serve as part of signal transduction cascades involving multiple proteins, which convey a redox-based signal, has not been shown. In this regard, it is important to note that the activation of kinases through oxidative inhibiton of phosphatases indeed constitutes redox-based regulation, but not signal transduction per se: signals are being *transduced* by phophorylation (kinases). By contrast, the activation of ASK by oxidation of Trx (discussed above) has the incipient fingerprint of oxidative signal transduction. In addition, it is key to understand whether responses are physiological signals or adaptive responses to stress. At present, the signaling function identified with NO biology, particularly S-nitrosylation, which is broad based, has not been replicated by alternative modifications, although recently much exciting headway suggest this is a real possibility.

Methodologies and to detect reversible cysteine oxidations in cells and tissues

Technical hurdles stem from the fact that cysteine oxidations relevant to signal transduction are reversed by commonly used reductants, such as beta-mercaptoethanol or dithiotreitol. Standard assays including, protein purifications, Western blots, (e.g. to assess kinase activity) and gel shifts (transcription factors binding to DNA), which routinely incude these reagents,

obscure the effect of oxidations. Mass spectrometric evaluation of complex mixtures also entails the use of reductants and changes in pH that destabilize Cys-based oxidations; further, an SNO does not often survive electrospray or MALDI (electrical current and ionization) treatments.

Thiol-specific alkylating reagents such as iodoacetic acid (IAA) or N-ethyl maleamide (NEM) which irreversibly bind to sulfhydryls, while not affecting oxidized cysteines [29,217–219] have opened the field to inquiry. The subsequent addition of reductant coupled to the use of a thiol-specific labeling agent such as biotinylated IAA or biotinylated NEM will then identify the oxidized Cys (Figure 3, top panel) [47]. This methodology, also referred to as “the thiol trapping technique” [220] has been successfully applied to cells in culture and demonstrated reversible oxidation and inactivation of tyrosine phosphatases in response to stimulation with growth factor receptors [29,116,117,219]. A similar strategy has been developed to detect S-nitrosylated proteins, widely known as the “biotin switch method” (Figure 3, middle panel) [56,221]. This approach also relies on thiol specific blocking and labeling reagents, but utilizes ascorbate to specifically denitrosylate S-nitroso bonds (formally a transnitrosation reaction, not a reduction). Indeed, Jaffrey and co-workers originally identified a number of proteins that were biotinylated following ascorbate treatment in wild type but not NOS1 knock-out mice, demonstrating a requirement of neuronal nitric oxide synthase (nNOS) in the ascorbate-dependent labeling of proteins, and establishing protein S-nitrosylation as a physiological event in the brain [56]. Although the specificity of the biotin switch method has been challenged due to concerns about the potential reactivity of ascorbate with disulfides or sulfenic acids [222, 223], those concerns were not well grounded. Reduction of disulfides, sulfenic acids and SNOs by ascorbate is not thermodynamically favorable (the transnitrosation reaction with SNO is not a redox reaction, hence the specificity). A recent comparative analysis of differentially oxidized or S-nitrosylated forms of proteins verified that the assay is highly specific for S-nitrosylated proteins; artifactual ascorbate-dependent signals were formed through exposure to indirect sunlight, likely induced by the semidehydroascorbate radical. One caveat with the biotin switch is the sensitivity: ascorbate does not effectively denitrosylate all S-nitrosothiols [127,224]. The coupling of the biotin switch coupled to SNO-photolysis (instead of ascorbate) provides an alternative means to detect S-nitrosylated proteins [127]. Overall, the technique has been a major advance, not only opening the field to outside disciplines but also serving to to verify earlier work through the 1990’s using more cumbersome techniques (mercury-coupled photolysis chemiluminescence and various other chemical and spectroscopic methods). Today, anti SNO-antibodies are also commercially available and have been used both in various histological analysis, Westerns, and immunoprecipitations [14,225,226].

Similar approaches have been taken towards the identification of S-glutathionylated proteins. In this endeavor, catalytically active Grx is used to reduce S-glutathionylated derivatives to the sylfhydryl group, followed by labeling of the newly reduced cysteine with a specific tag (Figure 3, bottom panel). This approach has been used in proteomic studies and identified a number of proteins targeted by S-glutathionylation, in addition to identification of specific sites of S-glutathionylation [154,227]. Related approaches to detect protein S-glutathionylation include the use of an antibody directed against GSH, GSH affinity purification, glutathione-S-transferase overlay, or the use of cellular labeling strategies with tagged and cell permeable glutathione, such as glutathione ethyl ester which becomes incorporated into proteins following an oxidative event and therefore is useful to detect proteins that are targets for S-glutathionylation [110,228–230]. It should be highlighted that the latter approach requires the use of an exogenous labeling agent (whose processing and cellular metabolism is not known) and does not allow detection of S-glutathionylated proteins that exist endogenously prior to the presence of GSH-labeling agent. The latter procedure also is problematic when evaluating S-glutathionylated proteins in tissues.

Thiol trapping methodologies have been adapted to visualize reversible cysteine oxidations in intact cells or tissues using microscopy approaches, in order to detect regional or kinetic changes in cysteine oxidations in subcellular compartments or in tissues *in vivo* [55]. Using these strategies, cells or tissues are first fixed in paraformaldehyde, permeabilized with triton-X100 in presence of the thiol blocking agent, NEM. Subsequently, S-nitrosylated cysteines are reduced with ascorbate followed by incubation with biotinylated NEM. Using streptavidin conjugated to a fluorophore, the location of the S-nitrosylated cysteine is then detected via confocal laser scanning cytometry [55] (Figure 4). Intriguingly, in cultured epithelial cells, substantial reactivity is detected in the nuclear compartment of cells, based upon co-localization with a DNA stain, and indeed the nuclear small GTPase Ran was demonstrated to be a target for S-nitrosylation [55]. Utilization of Grx as the reducing agent to detect S-glutathionylated cysteines yielded different staining patterns, with predominant staining in the cytoplasm or membrane ruffles, and marked increases in reactivity after exposure to H_2O_2, or expression of NOX1. The specificity of the Grx-catalyzed reduction for S-glutathionylated cysteines was highlighted using S-glutathionylated bovine serum albumin (BSA), a specific competitor in the reaction, vs. fully reduced or cysteinylated BSA, which did not affect the staining [100] (Figure 5).

Physiological relevance and pitfalls of models used to study redox signaling

Well over 3000 manuscripts have been published in the area of oxidants and “signaling”, but most of these manuscripts rely on the use of externally administered oxidants to cells in culture. Many investigators have raised concerns about the physiological relevance of those model systems. These concerns are warranted when excessive concentrations (>mM) of oxidants are utilized. However, the relevance of a particular experimental design depends on the nature of the hypothesis being tested [121]. If the experimental goal is to elucidate the nature of target cell responses triggered by high levels of reactive oxidants generated by inflammatory cells, exposing target cells to high amounts of oxidants probably represents a reasonable model system. However, if the experimental question is to determine how H_2O_2 or NO/SNO, generated from NOX or NOS, respectively under physiological settings, evoke physiological signaling, administration of NO or H_2O_2 externally, may produce spurious results. Exogenous agents are only useful to the extent they recapitulate the known physiology. As was highlighted in the previous section, oxidation events and antioxidant defenses are carefully balanced to allow a transient redox signaling event in precisely targeted locations, coordinated by the presence of adaptor proteins or chaperones that exist in signaling platforms within specific subcellular compartments. Given these considerations, it is unlikely that administration of oxidants extracellularly, can accurately model the signaling function of physiologically generated by H_2O_2 (irrespective of the concentration of oxidant applied) because different cysteines will be targeted. A hypothetical schematic that visualizes this concern is contained in Figure 6. On the other hand, the application of NO donors has recapitulated physiology more often than would be expected. It may be that NO cannot readily change cellular redox state and instead has been adapted to react with a subset of key thiols across a wide concentration range.

Assessment of the functional importance of cysteine oxidation *in vivo*

The relevance of cysteine oxidations of target proteins in the regulation of biological responses *in vivo* remains largely untested to date. Although many claims exist about the *in vivo* relevance of cysteine oxidation, most of the studies conducted so far have been limited to cells in culture. Many experiments, including work in our own laboratories, have relied on the expression of mutant constructs in which a reactive cysteine is mutated to an alanine or serine residue. In numerous cases, expression of the mutant construct produces a biological response that is distinct from the response triggered by the wild type construct, which have lead to conclusions

that oxidation of that particular cysteine is important *in vivo*. Compelling as this argument may be, conclusions can only be drawn with caution because of the stretched meaning of the "*in vivo*" scenario. Furthermore, in most studies, transfection of the mutated or wild type proteins occurred in a "transgenic" fashion where expression levels were not regulated or controlled, and the endogenous protein target was also present (experiments that were conducted in mouse embryonic fibroblasts derived from various knock-out mice would be the exception). Given these pitfalls, the question arises about how much we truly know about the functional relevance of cysteine oxidation events *in vivo*?

Drawing analogy to other areas of signal transduction, one approach used to overcome some of these pitfalls relies on transgenic mouse technologies. For example, in order to understand the impact of phosphorylation of a specific serine residue in a specific protein, the serine residue is mutated to a non-phosphorylatable residue, such as an alanine, and this way a "knock-in" mouse is created where the endogenous protein is replaced by the mutant version of the protein. This amino acid replacement strategy has the advantage that no interference exists with the endogenous protein, and that the mutant protein is regulated, and presumably expressed in the same manner as the wild type protein. An example of this strategy is the JunAA mice in which serines 63 and 73, the targets of phosphorylation of JNK, are mutated to alanines [231]. These mice have been instrumental in understanding the functional significance of phosphorylation of c-Jun in a number of pathophysiological settings [231–233]. Other examples of this strategy are available [234–237]. Similar strategies to evaluate the *in vivo* importance of specific cysteine oxidation targets are largely lacking, and only a few knock-in mice with mutated cysteine knock-ins have been reported. Rhodopsin is a target for palmitoylation at cysteine residues 322 and 323, and knock-in mice in which these sites were mutated to threonine and serine residues were reported to have enhanced shut-off phototransduction [238]. A mouse containing a single amino acid substitution (Cys249Trp) in the human Crumbs homolog-1 gene displayed in retinal degeneration [239]. Whether these phenotypes are linked to altered redox signaling remains to be determined. One knock-in mouse was generated to specifically address the functional significance of redox regulation *in vivo*. The protein, Ref-1 participates both in DNA repair pathways as well as in redox regulation. The redox function of Ref-1 involves reduction of oxidized cysteine residues within the DNA binding domains of several transcription factors, including Fos and Jun, and based upon elegant biochemical studies, cysteine 64 of mouse Ref-1 was identified as the redox catalytic site [240]. However, knock-in mice containing a cysteine-to-alanine point mutation into the Ref-1 gene displayed no evidence of aberrant redox regulation of c-Fos and c-Jun *in vivo*, questioning the *in vivo* relevance of the role of Ref-1 in redox regulation [241]. This finding was surprising because the biochemical analyses of the redox function of Ref-1 in cell culture models were very elegant. Intriguingly, a recent study suggested that other cysteines in Ref-1 can be S-nitrosylated [242], potentially explaining the lack of a phenotype in the Ref-1 cysteine 62 mutant mouse.

Conclusions and promises for the future

The area of redox-based signaling has arrived at an exciting juncture, bolstered by the concept that oxidative processes can be precisely orchestrated to evoke diverse functional responses. Newly recognized regulatory enzyme systems that operate together with classical antioxidant defenses serve to create independent modes of redox regulation within distinct cellular compartments. Furthermore, new technologies provide investigators with the opportunity, for the first time, to identify sites of oxidative modification within proteins with physiological and pathophysiological effects. While these studies are yielding exciting insights, NO/SNO remain more clearly linked with physiological signaling, and H_2O_2 with stress responses. These conclusions, however, have been derived largely from cell culture models and bioassays. Exploration of oxidative events in organisms will require implementation of large-scale

proteomic studies *in vivo*, including assessment of the spacial-temporal relationships between oxidative changes and organ (patho)physiology. Analyses of S-nitrosylation in NOS knockout animals with diverse pathophysiology has been an important step forward; by analogy, assessments of alternative S-oxidations in transgenic animals will no doubt be revealing. It will be exciting to watch developments that delineate the extent to which S-NO, S-OH and SSG are involved in health and disease.

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Abbreviations

ADAM
A Disintegrin and metalloprotease

AHR
Airway hyperresponsiveness

Alk
Alkylation

AMPA
alpha-amino-3-hydroxy-5-methylisoxasole-4-propionic acid

AP-1
Activator protein-1

ASK-1
Signal-regulating kinase-1

ARE
Antioxidant responsive element

Asc
Ascorbate

BSA
Bovine serum albumin

cGMP
Cyclic GMP

Cys-S-N-R
Sulfenyl amide

Cys-X-X-Cys
Common dithiol/disulfide active site motif

DUOX
Dual oxidase

EGF
Epidermal growth factor

ER
Endoplasmic reticulum

GADD
Growth arrest and DNA damage

GADPH
glyceraldehyde-3-dehydrogenase

Gpx
Glutathione peroxidase

Grx
Glutaredoxin

GST
Glutathione S-transferase

GSNO
S-Nitrosoglutathione

GSSG
Glutathione disulfide

HAT
Histone acyl transferase

HDAC
Histone deacetylase

HIF
Hypoxia-inducible factor

H_2O_2
Hydrogen peroxide

HSF
Heat shock factor

Hsp
Heat shock protein

IAA
Iodoacetic acid

IFN-γ
Interferon-gamma

LPS
Lipopolysaccharide

MAPK
Mitogen activated protein kinase

MKP
Mitogen activated protein kinase phosphatase

MMP
Matrix metalloproteinase

NAD
Nicotinamide adenine dinucleotide

NEM
N-ethyl maleamide

NF-κB
Nuclear factor kappa B

NH_3
Ammonia

NMDA
N-Methyl-D-Aspartate

nNOS
Neuronal nitric oxide synthase

NO
Nitrogen monoxide/nitric oxide

NOS
Nitric oxide synthase

NOX
Non-phagocytic oxidase

Nrf2
NF-E2-related factor 2

Nrx
Nucleoredoxin

$O_2^{\cdot}$
Superoxide anion

PDGF
Platelet derived growth factor

PDI
Protein disulfide isomerase

Prx
Peroxiredoxin

PTP
protein tyrosine phosphatase

PSSG
Protein-mixed disulfides/glutationylated cysteine Sirt, Sirtuin

SNO
S-nitrosylation, S-nitrosothiol, S-nitrosylated protein

SOH
Sulfenic acid

SO_2H
Sulfinic acid

SO_3H
: Sulfonic acid

S-Ox
: Oxidized cysteine

S-S
: Disulfide

TGF-β1
: Transforming growth factor-beta 1

TCF
: T cell factor

TLR
: Toll like receptor

TNF-R1
: Tumor necrosis factor receptor-1

Trx
: Thioredoxin

Srx
: Sulfiredoxin

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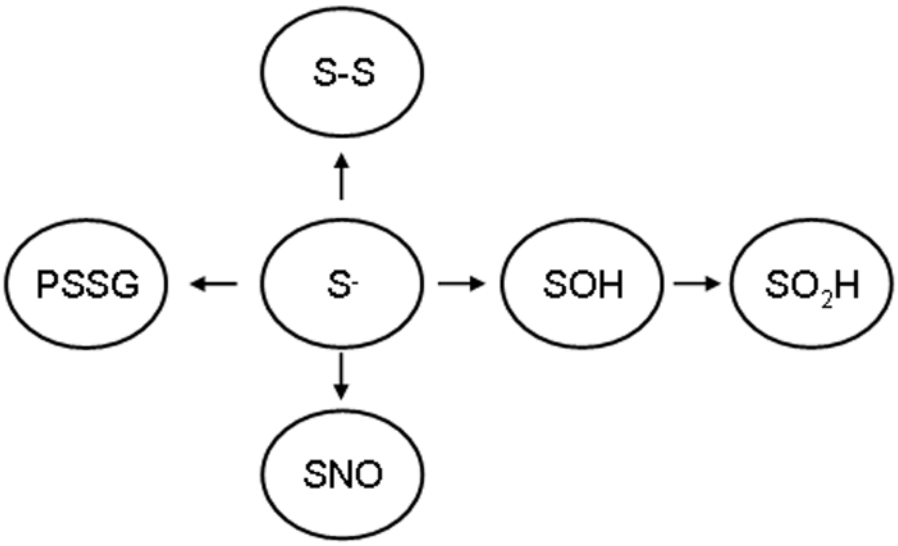

Figure 1.
Schematic overview of various reversible modifications of reactive cysteines (S^-). S-S; disulfide, PSSG; glutathionylation, SNO; nitrosylation, SOH; sulfenic acid, SO_2H; sulfinic acid. Note that the arrows are strictly included for an illustrative purpose, and do not indicate directionality.

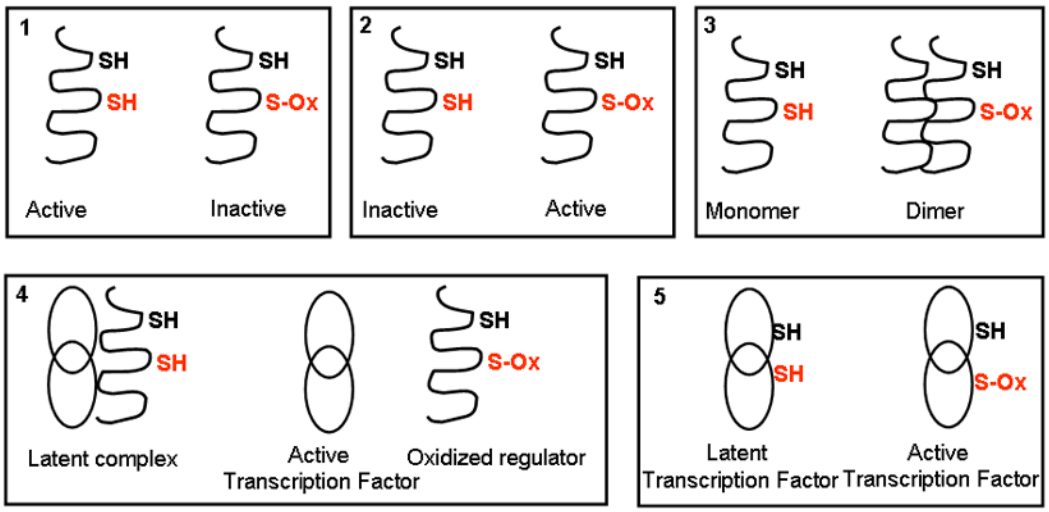

Figure 2.

Schematic representation of mechanisms of redox regulation.

Well described is the scenario where cysteine oxidation inactivates a signaling protein, such as a protein tyrosine phospatase (box 1) or caspases. Additionally, a protein with a signaling function can also be directly activated by oxidation of a critical cysteine residue (box 2). Examples of this scenario are Ras, ryanodine receptor, dynamin, SERCA, and Src tyrosine kinase ([50], for review). Another mechanism whereby cysteine oxidation elicits a signal is through control of multimerization (box 3), such as the dimerization of various heat shock or chaperone proteins or self-assembly of dynamin. Box 4 visualizes the situation wherein cysteine oxidation of a regulatory protein causes its dissociation of its partner, thereby activating the function of the partner. An example of this represents the dissociation of oxidized, thioredoxin from Ask1, leading to Ask1 activation, the dissociation from oxidized Keap-1 from NRF-2, leading the activation of NRF-2 as a transcription factor, or the dissociation of nNOS from glucokinase, leading to activation of the kinase [243]. Direct oxidation of transcription factors, may also be sufficient to mediate their activation or inhibiton through altered interactions with DNA (box 5). Examples include the activation of prokaryotic OxyR, which shifts from a “dimeric” to “tetrameric” interaction with DNA upon oxidation, and NF-κB (p50, p65) or cJun whose binding with DNA is inhibited by S-oxidation.

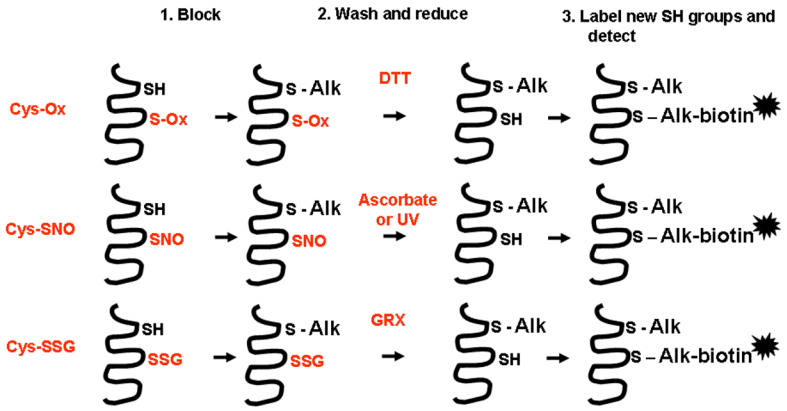

Figure 3.

Reduction strategies used to detect various cysteine oxidations. These thiol trapping or biotin switch procedures involve sequential steps of thiol blocking (1), a wash step to remove the chemical blocking agents, followed by a reduction step (2). This reduction step can employ non-specific chemical reagents e.g. dithiotreitol (DTT) to decompose sulfenic acids, disulfides, S-nitrosylated cysteines, or S-glutathionylated cysteines (top). Alternatively, the reduction step utilizes ascorbate or UV to reduce S-nitroso bonds (middle). GRX-catalyzed reduction (bottom) is used to reduce S-glutathionylated proteins. In a labeling step (3), the newly reduced sulfhydryl groups are reacted with a thiol specific labeling agent. Subsequently, by capturing the labeled cysteines, proteins can be identified using proteomic procedures. Labeled proteins can be precipitated, electrophoresed and blotted, and specific antibodies can then be used to detect targets. These methods can also be used to evaluate reversible cysteine oxidations *in situ*, in cells or tissues, using microscopy approaches (See Figures 4 and 5). Alk denotes a thiol specific alkylating agent, such as N-ethyl maleamide (NEM).

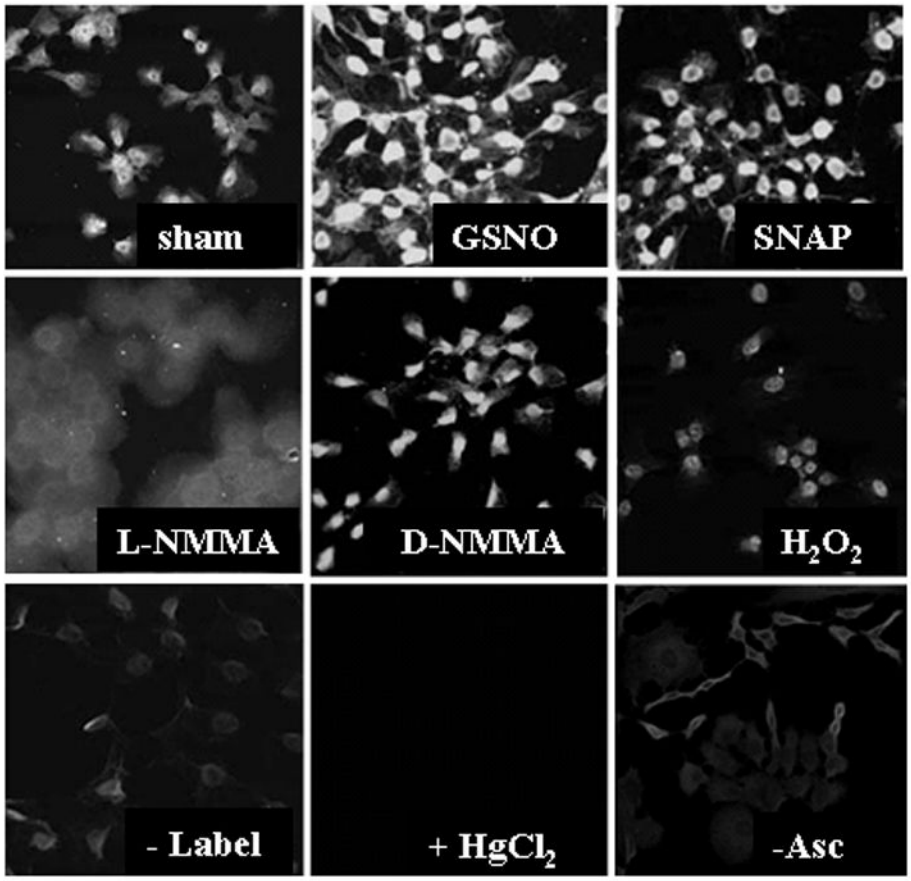

Figure 4.

In situ visualization of S-nitrosylated proteins in lung expithelial cells using confocal laser scanning cytometry. Cells were exposed to 1mM SNAP or GSNO for 1 hour, or to the NOS inhibitor, L-NMMA, or reagent control D-NMMA. As additional controls, the biotin-HPDP label (-label), or ascorbate (-Asc) were omitted, or $HgCl_2$ (which depletes SNO), was added before blocking to decompose S-nitrosothiols. As an additional control, cells were treated with H_2O_2 for 15 minutes (See [55] for experimental details). Reprinted from Nitric Oxide, Volume 11, In situ detection and visualization of S-nitrosylated proteins following chemical derivatization: Identification of Ran GTPase as a target for S-nitrosylation, Ckless K. et al., 216–227, Copyright 2004, with permission from Elsevier.

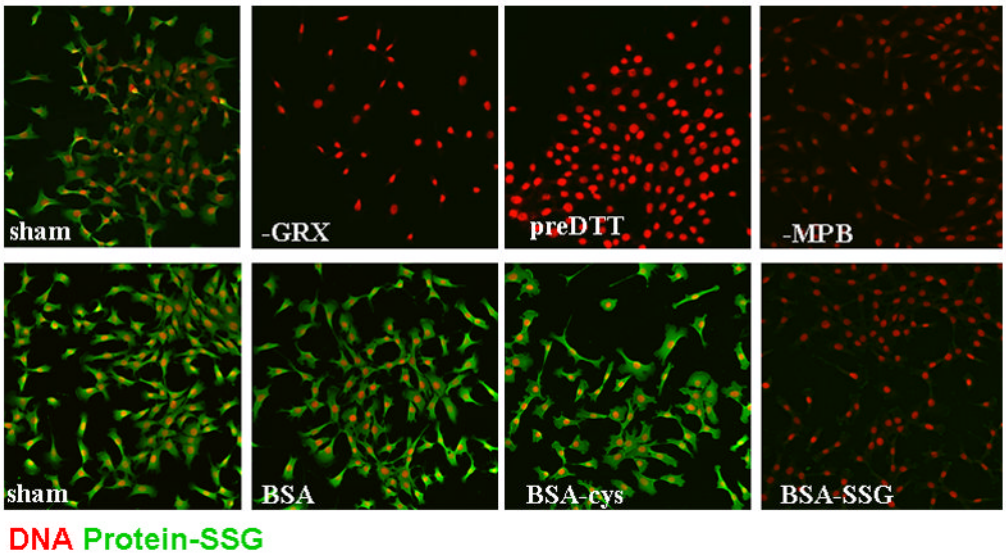

Figure 5.

In situ visualization of S-glutathionylated proteins in lung expithelial cells using GRX-catalyzed cysteine derivatization, and visualization via confocal laser scanning cytometry. Top panels: Lung epithelial cells were left untreated and as reagent controls, GRX (-GRX) or MPB (-MBP) were omitted out of the procedure. As an additional control, cells were treated with 1mM DTT prior to blocking with NEM (preDTT). Bottom panels: During the reversal of cysteine oxidations by GRX, fully reduced Bovine Serum Albumin (BSA), Cysteinylated BSA (BSA-cys) or S-glutathionylated BSA (BSA-SSG) were co-incubated in the reaction mixture to evaluate competition with endogenous substrates for GRX1-catalyzed reduction. (See [100] for experimental details). Protein S-glutathionylation is visualized in green, and nuclei are indicated in red. Reprinted from Biochimica et Biophysica Acta, Volume 1760, In situ detection of S-glutathionylated proteins following glutaredoxin-1 catalyzed cysteine derivatization. Reynaert N. et al., 380–387, Copyright 2006, with permission from Elsevier.

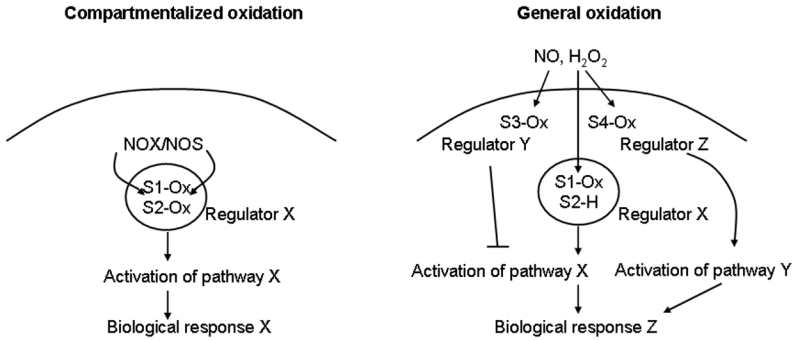

Figure 6.

Visualization of the differential impact of compartmentalized cysteine oxidation events, as compared to diffuse oxidation events on signal transduction cascades. The left panel represents a hypothetical schematic illustrating the compartmentalized cysteine oxidation events, S1-Ox, and S2-Ox, following the localized activation of NOX or NOS, which lead to activation of regulator X, which in turn evokes response X. The panel on the right represents diverse intracellular cysteine oxidation events S1-Ox, S3-Ox, and S4-Ox that occur in response to extracellularly encountered H_2O_2 or NO/SNO. This scenario leads to a different biological response (Z). Activation of Regulator X does not occur, because of lack of oxidation S2, and the oxidation of S3 which activates regulator Y, which in turn inhibits Pathway X. Instead, because of oxidation event S4, activation of regulator Z occurs, leading to activation of pathway Y, and response Z. The scenarios illustrated are strictly hypothetical.

Table 1

Enzymes controlling reversible cysteine oxidations

Family	Target oxidation	Designation	Reference
Thioredoxin	Disulfides	SS	[83]
Glutaredoxin	S-glutathionylated cysteines	PSSG	[47]
Bacterial glutaredoxin*1	Disulfides	SS	[83,99]
Protein disulfide isomerase	Disulfides	SS	[156]
Sulfiredoxin	Peroxiredoxin sulfinic acids	Prx-SO_2H	[65]
GSNO reductase	S-nitrosothiols*2	SNO	[20]

*1 In addition to the monothiol reduction of glutathionyl disulfides, bacterial Grx also reduces low molecular weight disulfides through a dithiol mechanism [83,99]. However mammalian GRX displays marked substrate specificity towards S-glutathionylated proteins [100,101]. Thus, the major target for Grx in mammalian cells in physiological setting is S-glutathione mixed disulfide (PSSG).

*2 GSNO reductase decomposes low molecular weight S-nitrosothiols, thereby indirectly affecting S-nitrosylated protein content.