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Forum Review

Regulation of Signal Transduction Through Protein Cysteine Oxidation

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ABSTRACT

The production of reactive oxygen species (ROS) accompanies many signaling events. Antioxidants and ROS scavenging enzymes in general have effects that indicate a critical role for ROS in downstream signaling, but a mechanistic understanding of the contribution of ROS as second messengers is incomplete. Here, the role of reactive oxygen species in cell signaling is discussed, emphasizing the ability of ROS to directly modify signaling proteins through thiol oxidation. Examples are provided of protein thiol modifications that control signal transduction effectors that include protein kinases, phosphatases, and transcription factors. Whereas the effects of cysteine oxidation on these proteins in experimental systems is clear, it has proven more difficult to demonstrate these modifications in response to physiologic stimuli. Improved detection methods for analysis of thiol modification will be essential to define these regulatory mechanisms. Bridging these two areas of research could reveal new regulatory mechanisms in signaling pathways, and identify new therapeutic targets. *Antioxid. Redox Signal.* 8, 1819–1827.

INTRODUCTION

Reactive oxygen species (ROS) are produced in response to a number of inflammatory signaling mediators, including TNFα (46, 59), IL-1β (46, 77), IFNγ (36, 57), T cell receptor ligation (14, 73), Toll-like receptor signaling in response to LPS (3, 55), and CD40 ligation in B cells (24, 37). Evidence has generally suggested that ROS are not simply by-products of signaling events, but rather act as second messengers that are required for the downstream signaling effects. However, mechanism(s) by which ROS are sensed and translated by the signaling machinery have remained elusive.

Independently, mechanisms of control of cell signaling through direct, targeted, and reversible oxidation of cysteine residues in proteins have been outlined in several systems. Similar to the regulation of protein function by phosphorylation, oxidation of cysteine residues results in conformational, structural, and direct catalytic consequences on targeted signaling proteins. Yet, while clearly detectable in experimental systems, the observation of these modifications in response to physiologically relevant signaling stimuli has proven more difficult.

GENERATION OF ROS IN RESPONSE TO STIMULATION

ROS generation was first observed as part of the "respiratory burst" in phagocytic macrophages. Detection of ROS in cells often relies on use of fluorescent dyes that display altered fluorescent properties when oxidized. As detection approaches have improved, it has become evident that many cell types generate ROS in response to a variety of extracellular stimuli, though at levels far below that seen in the phagocytic macrophage. Growth factors including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), and inflammatory cytokines such as TNF-alpha and IL-1 are among the best-studied stimuli of ROS generation (for review, see Ref. 70).

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Indirect methods have been used to demonstrate that the ROS are also critical participants in the downstream signaling processes. For example, antioxidant chemicals [*e.g.*, *N*-acetyl cysteine (NAC)] or overexpression of scavenger enzymes [*e.g.*, catalase and superoxide dismutase (SOD)], can interfere with ROS and block many or all of the downstream signaling effects. Specifically, pretreatment of cells with superoxide scavengers such as NAC or pyrrolidine dithiocarbamate (PDTC) inhibits activation of NF-κB in response to TNFα treatment (47, 67). In contrast, these agents fail to suppress AP-1 activation by TNFα, demonstrating specificity of the oxidative events (67). Similarly, overexpression of MnSOD (4) similarly blocks TNF signaling pathways, further supporting the critical role of ROS in TNF induced signal transduction.

Production of ROS by phagocytic macrophages involves a complex of proteins that is located at the plasma membrane, and is referred to as the NADPH oxidase (phox) complex (for review, see Refs. 6 and 54). This complex was long thought to be specific to macrophages, but recent work has demonstrated that other cell types have NADPH oxidase systems comprised of some components of the macrophage phox system, and that are capable of producing ROS via a similar mechanism. For example, lipopolysaccharide (LPS) signaling through Toll-like receptor 4 (TLR4) involves Nox4, a phox enzyme similar to the Nox2/gp91phox enzyme present in the macrophage phox complex (51). This study demonstrated direct binding of TLR4 to Nox4, and also that depletion of Nox4 blocked LPS-induced NF-κB activation. A separate study showed that TLR4 was required for generation of ROS in response to LPS, and was also required for the assembly of a signaling complex that activated a downstream kinase (the mitogen activated protein kinase, p38) and that participated in innate immunity (44). Together, these studies begin to define a complete system in which ROS are generated, sensed, and translated into the LPS response.

Other stimuli induce ROS through a pathway involving mitochondria. Demonstrating this, disruption of mitochondrial electron transport interferes with TNFα signaling responses including apoptosis and NF-κB dependent gene expression (25, 27, 64, 65, 68). Mitochondria-targeted antioxidants can promote TNFα-induced apoptosis by delaying NF-κB activation (29), suggesting that mitochondria-derived ROS are important in the appropriate regulation of NF-κB-dependent survival gene expression. Despite these results, the mechanism of ROS generation by mitochondria in response to TNFα remains unclear.

GENERATION AND NEUTRALIZATION OF ROS

The importance of ROS in cell signaling can be inferred from the complex regulatory pathways that have evolved to control them. High levels of ROS can harm cellular macromolecules such as proteins, lipids, and DNA. The presence of ROS is often correlated with subsequent cell death, so it has widely been held that apoptotic death is a direct consequence of widespread and nonspecific damage resulting from ROS. More recent work has suggested that in at least some instances, ROS participate in a controlled, active signaling process to promote, and sometimes to prevent, apoptosis. Cells have evolved many layers of protective defenses to neutralize ROS, protecting the cell from potentially toxic effects.

We will mainly consider signaling processes affected by superoxide and the less reactive hydrogen peroxide (H_2O_2). Superoxide and peroxides are interconnected by a complex network of enzyme systems that serve to interconvert and neutralize them. We refer interested readers to relevant reviews (18, 75), and for a discussion highlighting the interconnected nature of these pathways, we note a review by Nordberg and Arner, which describes the wide-ranging effects of pharmacological interference with a single enzyme in the system, thioredoxin reductase (49).

Superoxide is continually generated within mitochondria, as a result of electron leakage from the electron transport chain. It is generated in larger quantities by NADPH oxidase complexes, during phagocytosis and other cell signaling response, as discussed above. Superoxide is not highly reactive and cannot cross cellular membranes, so in most cases, its potential effects are restricted to a single intracellular compartment. However, through the action of superoxide dismutase, superoxide is rapidly converted to hydrogen peroxide that can readily penetrate membranes. Hydrogen peroxide can then be neutralized through action of catalase that converts two H_2O_2 molecules into molecular oxygen and water. However, since catalase expression is predominantly restricted to the peroxisome, intracytoplasmic hydrogen peroxide may exhibit a relatively long lifetime. Within the cytosol and the mitochondria, other antioxidant enzymes, such as glutathione peroxidases and peroxiredoxins, are responsible for protection from peroxides via reactions that consume glutathione. Effectively, these reactions result in oxidation of protein- or small molecule-thiols in exchange for reduction of hydrogen peroxide.

The most abundant cellular antioxidant is the cysteine-containing tripeptide glutathione (GSH). GSH is present in millimolar concentrations in mammalian cells. Spontaneous and enzyme-catalyzed conversion of reduced GSH to oxidized GSH (*i.e.*, GSSG) serves as a redox buffer system within cells, consuming hydrogen peroxide in the process. Oxidative stress of many sources is generally indicated by a lower ratio of reduced to oxidized glutathione (GSH:GSSG) (61). The GSSG can then be reduced by the glutathione regenerating system that includes the glutathione reductase enzyme, and obtains protons from NADH or NADPH.

In addition to its role as a redox buffer and in direct neutralization of ROS, GSH can form mixed disulfides with protein thiols. Because the GSH tripeptide is bulky and charged, GSH adducts result in alterations protein function as discussed below. While GSH may induce a functional alteration in modified proteins, reversible glutathionylation protects protein thiols from higher order and therefore irreversible oxidation events. Upon reestablishment of a more normal intracellular redox environment (*i.e.*, a restored GSH:GSSG balance) glutathionylation might be easily reversed, restoring normal protein function. Protein glutathionylation can be reversed by another class of glutathione regulating enzymes, the glutaredoxins (for a recent review, see Ref. 66). In this manner, the abundance of intracellular GSH, or other regulators of glutathionylation, might serve as a sensor of the intensity of an

oxidative insult, and act as a functional switch. Below a specific threshold level, there might be little damage to signaling proteins, whereas above that level, oxidative damage could have functional effects on signaling proteins.

The mammalian thioredoxin system provides a separate redox buffering system. Thioredoxin (TRX) has a long history in control of signaling function. Thioredoxin is a small protein that functions as a disulfide oxidoreductase. The TRX proteins contain a catalytic site composed of two closely-spaced cysteine residues in a highly conserved active site sequence. One of these two cysteines exists in thiolate ($R\text{-}S^-$) form (33) and can be readily oxidized to sulfenic acid, which then reacts with the second cysteine thiol to form an intramolecular disulfide bridge (28). This disulfide is then catalytically reversed by thioredoxin reductase to regenerate reduced TRX, in a process that requires NADPH (18). A similar catalytic mechanism is used by the peroxiredoxins to convert H_2O_2 in the glutathione-dependent pathways above [(63), for recent review, see Ref. 76]. Importantly, while TRX reacts only slowly with H_2O_2, one of the dominant functions of TRX activity is regeneration of the active site of peroxyredoxin (PRX), highlighting the interconnectedness of the antioxidant pathways. In addition, TRX has been proposed to act on protein disulfides in several transcription factors including p53 (52), NF-κB (45), and AP-1(62), working to enhance their DNA binding activity.

THIOL MODIFICATION OF SPECIFIC CYSTEINES

With several ROS-neutralizing defenses in cells, it is perhaps surprising that an ROS molecule can persist long enough to play a significant role in signaling. As a parallel to this situation, Forman *et al*. (19) compared the possible signaling effects of ROS to the activation of protein kinase A (PKA) by the small and easily inactivated molecule cyclic AMP. Cyclic AMP is able to activate a small pool of PKA despite the presence of ample phosphodiesterase to neutralize it. This analogy suggests the importance of intracellular localization of ROS in their signaling capabilities; modest levels of ROS generated in response to a physiological stimulus might affect only a small pool of protein located proximal to the source. This localized response could also contribute to the difficulty in detecting oxidative modification of protein targets since they may impact only a small fraction of the protein in an otherwise unaffected majority.

A second determinant of specificity relates to protein targets. In theory, all cysteine thiols could be targets for redox modification, and perhaps many are indeed modified with high levels of ROS. However, for thiol modification to be important for cell signaling, there must be determinants of specificity that enable a small subset of cysteines to become modified and to effect specific changes in protein function. As the examples discussed below will illustrate, thiol modifications are specific targeted events that generally affect single cysteine residues on a given protein.

Two factors appear likely to contribute to the phenomenon of specificity: the accessibility of a given cysteine and its inherent reactivity. Accessibility can be reflected experimentally by differential sensitivity to thiol modifying agents such as *N*-ethyl malemide (NEM) or iodoacetamide (IAA), which have been shown to preferentially modify cysteines located at the surface over those buried within a nondenatured protein. It is likely that ROS, though much smaller than these chemical thiol modifiers, may be somewhat sensitive to the accessibility of a given cysteine residue.

Second, the reactivity of a given cysteine may be influenced by contributions from the surrounding amino acids. In the overall reducing environment present in the cytoplasm, the majority of cysteines exist as sulfhydryls (R-SH). Particularly in the case of H_2O_2 signals, sulfhydryls are nonreactive. Only thiolate anions ($R\text{-}S^-$) are capable of directly reacting with H_2O_2. The existence of thiolate anions within a cell is dependent on contributions of the surrounding amino acids to thiol ionization, with a prevalence of basic amino acids thought to contribute to reactivity. Alternatively, the local microenvironment of a given cysteine residue, dictated by the tertiary structure within a folded protein, may contribute to the tendency of that cysteine to exist in the reactive thiolate form. For a more extensive discussion of specificity in thiol reactions, we refer the readers to recent review articles (19, 21) that focus on this topic. Overall, chemical reactivity secondary to the local amino acid environment is likely to contribute to the modification of certain cysteine residues in preference to others.

THIOL OXIDATION IN CELL SIGNALING

ROS are clearly required for many signaling responses. The literature is replete with reports that conclude that "ROS activate (or inhibit) signaling protein X". In most cases, this indicates only that protein X is functionally activated or otherwise altered when ROS are present, and/or that ROS generation by an upstream stimulus is required for the effect. In other cases, these conclusions are drawn from coincident generation of ROS and activation of protein X. In most cases, the actual mechanism(s) by which ROS are sensed, interpreted, and translated into downstream signaling responses have remained elusive. We (10, 11) and others (17, 18, 21, 50, 70) have proposed that direct modification of thiols in pivotal signaling proteins plays a central role in this process.

Regulatory thiol oxidation of cell signaling proteins could serve several different purposes in signal transduction. Typically, pro-oxidative events correlate with activation of downstream signaling cascades; studies with antioxidants and ROS scavenging enzymes support a requirement for ROS in activation of these pathways. Perhaps paradoxically, as the examples discussed below will illustrate, many of the direct thiol oxidation events characterized to date are inhibitory, blocking the activity of the target molecule. Inhibitory modification of kinases would be expected to inhibit downstream signals, so these events may play a crucial role in feedback regulation of pathways, as a means of terminating signals. Contrarily, inhibition of protein phosphatases may indeed contribute to the activation of downstream pathways, by blocking the dephosphorylation of signaling proteins and enhancing the signaling

response. Many of the targets for direct thiol oxidation are integral players in apoptotic signaling, and the effects on their activity are consistent with ROS generation inducing a regulated apoptotic program. This is particularly provocative given the concomitant role of mitochondria in both ROS generation and apoptotic signaling.

ROS-MEDIATED THIOL OXIDATION

During oxidative stress response, cysteine residues may be directly oxidized to sulfenic acid (R-SOH), or further oxidized to higher order sulfinic (RSO_2^-) or sulfonic ($R\text{-}SO_3^{2-}$) acids; they may form intra- or intermolecular disulfide bonds, or participate in a mixed disulfide bond with a small intracellular thiol such as glutathione, in a process referred to as glutathionylation, (or synonymously, glutathiolation or *S*-thiolation). In some cases, thiol modifications are interdependent. For example, oxidation of cysteine to sulfenic acid (a relatively unstable modification) is sometimes followed by "resolution" by reaction of the sulfenic acid with a nearby cysteine residue to form a more stable disulfide bond. The resolving cysteine may be contributed from within the same protein, by a second protein, or by glutathione, leading to different ultimate modifications. In each case, experimental observation of the transient sulfenic acid modification would be difficult.

EXAMPLES OF PROTEIN REGULATION BY CYSTEINE OXIDATION

Below, we discuss several protein targets of regulated thiol modification that have clear connections to cell signaling in mammalian systems. In most cases, the role of these thiol modification events in the regulation of signaling response downstream of physiologically relevant stimuli is still under investigation. These examples are illustrated in Fig. 1.

SAPK/JNK

The stress activated protein kinase (SAPK, also known as jun N-terminal kinase (JNK)) pathway is activated by diverse stimuli including UV light, osmotic shock, and inflammatory cytokines. A number of the participants in this signaling cascade are targeted by thiol modifications. The SAPK protein kinase itself is regulated by an inhibitory interaction with a member of the glutathione S transferase family, GSTpi. Stimulation with UV light or hydrogen peroxide leads to oligomerization of GSTpi, releasing SAPK, with an increase in activity (2). Conversely, we have observed that SAPK is inhibited by spontaneous thiol modification *in vitro* in buffers lacking reductants, or in cells treated with strong oxidants such as menadione or hydrogen peroxide. This modification can be reversed by exposure of purified protein to reducing agents

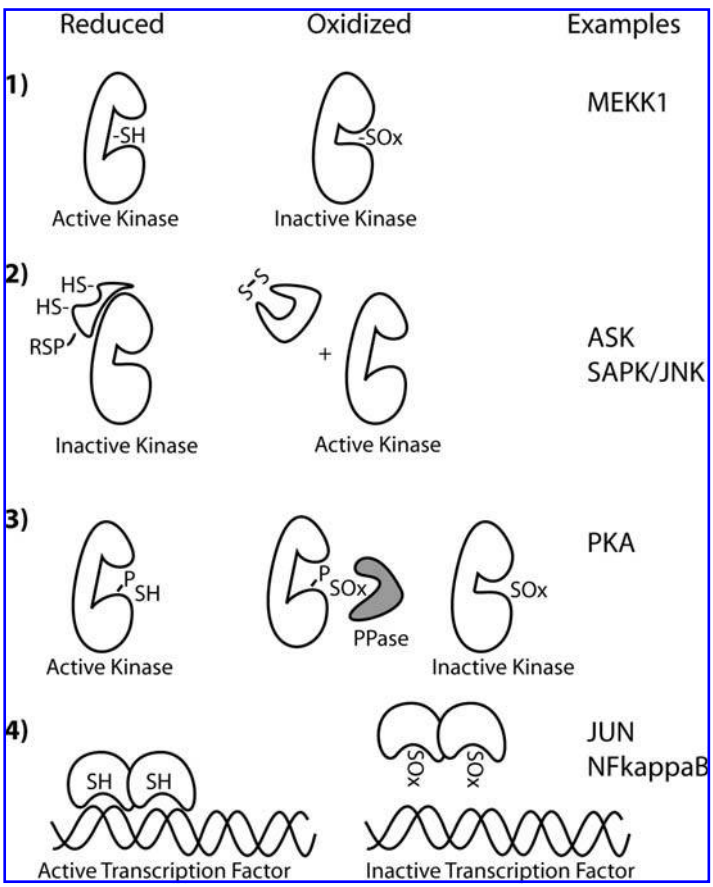

FIG. 1, Mechanisms for modulation of protein function by oxidation. Examples of proteins functionally modified by cysteine oxidation through several mechanisms. **1**) MEKK1 contains a cysteine residue (SH) within the ATP binding domain. Oxidation of the cysteine (SOx) interferes with kinase activity. **2**) Kinase activity is inhibited by interaction with a redox-sensing protein (RSP) such as thioredoxin. ROS generation leads to oxidation of the RSP and dissociation of the complex, releasing active kinase, exampled by ASK and SAPK/JNK. **3**) Protein kinases are commonly phosphorylated within the activation loop (P). Oxidation of PKA on an adjacent cysteine residue increases susceptibility to a protein phosphatase (PPase), leading to dephosphorylation and inactivation. **4**) Transcription factors activate transcription through DNA binding. Oxidation of a cysteine residue in the DNA binding domain interferes with DNA binding, preventing transcriptional activity.

such as dithiothreitol (unpublished observation), evidence that it is due to a direct reversible thiol modification. These observations suggest that SAPK may be a target for regulated thiol modification during responses that generated ROS. Importantly, the inhibition of SAPK by thiol modification is not reflected in a change in phosphorylation of the activation loop. Therefore, using anti-phosphoSAPK antibodies alone to measure SAPK activity would fail to detect inhibition resulting from oxidation effects.

MEKK1 and ASK1

Two upstream activators of the SAPK pathway are also regulated by oxidative stress. We have demonstrated that the MEKK1 protein kinase, a MAP3K level upstream kinase in the SAPK cascade, is inhibited by site-specific glutathionylation of a critical cysteine residue in the ATP binding domain (10). Mutation of this cysteine residue results in an active kinase that is no longer inhibited by oxidative stress. MEKK1 is activated by TNFα and CD28 co-receptor signaling (71, 74), as well as by microtubule destabilizing drugs such as nocodazole and paclitaxel (38, 78). Thiol oxidation of MEKK1 is seen in response to relatively strong oxidative stimuli (*i.e.*, menadione or H_2O_2) and thiol modification may serve as a means of feedback inhibition of kinase signaling similar to the NF-κappaB pathways below (in which MEKK1 has also been proposed to play a role).

A second MAP3K in the SAPK signaling pathway is also regulated by thiol modification, though less directly. The apoptosis signaling kinase 1 (ASK1) is inhibited by interaction with thioredoxin (TRX) (41, 56). As discussed above, TRX is a small protein that functions as an ROS scavenger. In this case, TRX inhibits ASK1 by physical binding. In response to H_2O_2 or TNF stimulation, TRX is oxidized on the active site cysteines, promoting dissociation from ASK1, and resulting in activation of the kinase. In addition, ASK1 is similarly inhibited by interaction with other redox active proteins including glutaredoxin (GRX) and glutathione *S*-transferase mu (GSTmu). The interaction with GSTmu is disrupted by heat shock in a mechanism that is insensitive to NAC, suggesting that it might not be a redox regulated event (15). In contrast, the complex between ASK1 and GRX is redox sensitive and dissociates in response to glucose deprivation (69). Taken together, these results suggest that oxidative stress would result in activation of ASK1, potentially initiating or promoting an apoptotic program. This may be further enhanced by the inhibition of survival signaling pathways (*e.g.*, NF-κB, MEKK1) that is likely to accompany ROS generation.

Protein kinase A

The cyclic AMP-dependent protein kinase (cAPK or PKA) is activated in response to elevated cAMP levels. Following treatment of cells with diamide, PKA is targeted for site-specific glutathionylation at a cysteine residue near the active site of the kinase, cysteine 199 (30). Similar to MEKK1, mutation of this cysteine results in a kinase that is resistant to glutathionylation and inhibition in experimental systems. Importantly, this cysteine is located near the critical phosphorylation site in the active site of PKA, threonine 197. In a follow up report, Humphries *et al*. demonstrated that oxidation of the cysteine at position 199 enhanced the dephosphorylation and inactivation of PKA (31). Therefore, in contrast to MEKK1, where inhibition appears to result from interference with ATP binding, glutathionylation of PKA promotes dephosphorylation as a mechanism of inhibition. Identification of an endogenous signaling pathway that utilizes this glutathionylation and dephosphorylation mechanism for regulation of PKA awaits further study.

PKC family

Several members of the PKC family have been identified as targets for thiol modification. PKC epsilon, a novel PKC with proposed roles in growth, survival, and inflammatory signaling, is inhibited by thiol modification. Glutathionylation, cysteinylation, and disulfide bond formation have all been detected, depending on the experimental approach (8, 9). In the same experiments, a second novel PKC family member, PKC delta, not only retained activity but was modestly activated by thiol modification, in this case a cysteinylation event. Consistent with the apoptosis theme from above, activation of a pro-apoptotic PKC (PKC delta) along with inhibition of the survival signaling PKC epsilon could contribute to initiation or promotion of apoptosis, particularly in combination with the described effects of thiol modification on other targets.

Akt/PKB

The Akt protein kinase (also known as PKB) is closely implicated in cell survival signaling, and participates in both nutrient and growth factor signaling. Under oxidative stress conditions, Akt forms an intramolecular disulfide bond that targets the protein for dephosphorylation by enhancing its interaction with protein phosphatase 2A. Importantly, the disulfide bond in Akt can be reversed by glutaredoxin *in vitro*, and the dephosphorylation of Akt is prevented by overexpression of GRX, suggesting that this modification represents a redox-regulated reversible event (48). Inhibition of Akt would suppress its survival functions, consistent with the proposed role of ROS in promoting apoptosis.

Protein phosphatases

The effects of protein kinases are reversed by the activity of protein phosphatases. Provoked by the critical cysteine residue at the active site, protein phosphatases were among the earliest characterized targets of inhibition by thiol modification. In contrast to most of the targets described above, oxidation and inhibition of protein phosphatases has been observed in response to physiologically relevant signaling by growth factors (*e.g.*, EGF) (39). The best characterized target, protein tyrosine phosphatase 1B (PTP1B), is inhibited through a transient sulfenic acid oxidation event, observed by exposing purified protein to hydrogen peroxide *in vitro* (13). The sulfenic acid is then resolved by reaction with an adjacent amino acid to form a more stable sulfenyl amide bond (58, 72). In independent experiments, others have suggested that PTP1B can also be inhibited by glutathionylation of the active site cysteine, that also likely proceeds via a sulfenic acid intermediate (5). Both of these oxidation events would serve to protect the active site cysteine from higher order oxidation to sulfinic or sulfonic acid, events that would be mostly irreversible.

Although PTP1B is the best studied, several other protein phosphatases have been identified as targets for inhibition by thiol modification. In most cases, inhibition involves a sulfenic acid oxidation event, followed by resolution through reaction with a nearby cysteine residue to form a more stable (but still reversible) disulfide bond. These targets include the PTEN tumor suppressor (a lipid phosphatase) (40), the cell cycle regulatory cdc25c phosphatase (60), and the low molecular weight protein tyrosine phosphatase (LMW-PTP) (7).

By utilizing ROS, growth factors and inflammatory mediators may transiently inhibit the activity of a broad range of protein phosphatases that would otherwise act in an antagonistic manner to suppress downstream signaling. Therefore, inhibition of protein phosphatases could be at least part of the mechanism by which ROS generation is sensed and translated into activation of signaling pathways.

Transcription factors

A central mediator of immune system function is the NF-kappaB transcription factor. NF-κB is involved in both cellular responses to and expression of an array of genes. As an example, we will focus on TNF-α, but cell responses can be generalized to many other signaling pathways. NF-κB is best characterized as a heterodimer of two proteins, p50 and p65. This dimer is sequestered in the cytoplasm in complex with an inhibitory protein, IkB. In response to stimulation by TNF, IkB is phosphorylated on two serines near its amino terminus by the IkB kinase (IKK), which targets IkB for ubiquitination and degradation by a proteasome-mediated pathway. Degradation of IkB releases NF-κB to translocate to the nucleus and activate expression of an array of target genes, including IkB-α itself, resulting in negative feedback regulation of NF-κB signaling. For a more complete discussion of the NF-κB pathway, we refer the reader to reviews dedicated to this topic (23, 23).

How ROS impact TNF signaling is not well understood. As discussed above, it is clear that TNF-α results in cytoplasmic generation of ROS and interference with ROS abolishes most downstream signaling events, including activation of NF-κB-dependent gene expression. The known protein thiol modifications within this cascade are inhibitory of protein function, and are therefore inconsistent with a role in the activation phase of the NF-κB pathway. For example, IKK is subject to a poorly characterized inhibitory oxidation event (35). As well, the DNA binding activity of the p50 NF-κB protein is inhibited by glutathionylation or sulfenic acid oxidation of a critical cysteine in the DNA binding domain (53). All of these events may serve as negative feedback mechanisms to down-regulate NF-κB activation, but all are inconsistent with a role in the activation of this pathway. Therefore, the mechanism by which ROS act as obligate participants in activation of NF-κB (or other TNF signaling responses) remains an open question.

The *c*-jun transcription factor, a nuclear target of the SAPK pathway, is also sensitive to redox regulation. Similar to NF-κB p50, the DNA binding domain of *c*-jun contains a critical cysteine residue that is glutathionylated *in vitro* in response to altered GSH/GSSG ratios, reducing DNA binding activity (34). The inhibitory effect on DNA binding could translate into abrogated transcriptional response in intact cells during cell stress.

Ras

Often a focus for study due to its oncogenic properties in tumorigenesis, the small GTPase and oncoprotein Ras has a long appreciated role in cell signaling (for review, see Ref. 20). Ras is glutathionylated following hydrogen peroxide treatment *in vitro* or treatment of intact cells with diamide (42), as well as during reperfusion in a model of ischemic heart (16). In experimental systems, Ras can be modified on four different cysteines, one of which lies in the C-terminal CAAX box common to all Ras-family members, where farnesylation normally occurs (42). Oxidation of this cysteine might prevent farnesylation and could interrupt aspects of Ras function that are dependent on membrane localization. Conversely, glutathionylation of Ras at Cys 118 is induced by angiotensin II signaling, and increases Ras activity (1). Thus, separately controlled and molecularly distinct oxidations regulate Ras in a complex manner. Importantly, these studies with Ras also provide one of the best examples of identification of oxidative modification resulting from a physiologic stimuli.

REACTIVE NITROGEN SPECIES

All of the thiol modifications discussed above are the result of reactive oxygen species leading to oxidation events on target cysteines. However, protein thiols are also sensitive to modification in response to nitric oxide and other reactive nitrogen species (RNS), via a process referred to as *S*-nitrosylation. RNS are crucial mediators of many signaling events, particularly during inflammatory responses (12, 32). The role and targets of *S*-nitrosylation are beyond the scope of this review, and have been discussed extensively elsewhere (26, 43).

FUTURE DIRECTIONS

Several questions remain regarding the role of ROS-dependent thiol modifications in the control of cell signaling. Most significantly, the ability to detect these modifications as they occur in response to physiologically relevant stimuli is tremendously important in understanding their contribution to global cell signaling responses. Currently available technology is only beginning to approach the ability to detect minor modified species in a pool of mostly unmodified protein. Until this goal is attained, the mechanistic dissection of these signaling pathways will be near impossible. Defining *specific* events is a current challenge as well. How general events such as hydrogen peroxide generation are translated into localized and even sequence-specific signaling events such as cysteine oxidation and secondary consequences such as protein phosphorylation and gene expression remain unclear. Perhaps even more important, the potential role of cysteine oxidation in regulation of signal transduction must be appreciated. Cysteine oxidation can effect covalent changes that alter protein secondary or tertiary structure, and can introduce localized protein charges similar to protein phosphorylation. It might be concluded that historically, only because protein phosphorylation could be easily detected through the use of radiophosphate was the significance of protein phos-

phorylation understood before control of cell signaling by cysteine oxidation. Uncovering the targets of oxidation control and the enzymes responsible for these alterations will enable understanding of this important regulatory mechanism.

ABBREVIATIONS

AP-1, activating protein 1; ASK1, apoptosis signal-regulating kinase 1; cAPK, cyclic AMP-dependent protein kinase; EGF, epidermal growth factor; GRX, glutaredoxin; GSH, glutathione (reduced); GSSG, oxidized glutathione; GST, glutathione *S*-transferase; H_2O_2, hydrogen peroxide; IAA, iodoacetimide; IFN, interferon; IKK, IkappaB kinase; IL-1, interleukin 1; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MEKK, MAPK/ERK kinase 1; NAC, *N*-acetyl cysteine; NEM, *N*-ethyl-malemide; Nox4, NADPH oxidase 4; PDGF, platelet-derived growth factor; PDTC, pyrrolidine dithiocarbamate; PKA/B/C, protein kinase A/B/C; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homology deleted on chromosome 10; PTP1B, protein tyrosine phosphatase 1B; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAPK/JNK, stress activation protein kinase/jun N-terminal kinase; SOD, superoxide dismutase; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRX, thioredoxin.

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